Neuron-specific Expression of the Human Dopamine $\beta$-Hydroxylase Gene Requires Both the cAMP-Response Element and a Silencer Region*

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Dopamine $\beta$-hydroxylase (DBH), the enzyme catalyzing the conversion of dopamine to norepinephrine, is specifically expressed in adrenergic and noradrenergic neurons in the central nervous system. DNase I hypersensitive sites were found in the 5'-flanking region of the DBH gene in noradrenergic human neuroblastoma SK-N-BE(2)C cells, but not in DBH-nonexpressing HeLa cells. We report here that the 4.3-kilobase upstream sequence of the human DBH gene confers cell type-specific expression as assessed by transient expression assay. Furthermore, deletional and mutational analyses revealed two genetic regulatory elements required for the regulation of cell type specificity. First, deletion of the cAMP-response element (CRE) abolished >95% of the transcriptional activity by the DBH upstream promoter, thus implicating the CRE as an essential positive genetic element. Second, deletion of a region between -490 and -263 base pairs resulted in 10-fold increase of reporter gene activity only in HeLa cells, indicating that this region contains a cell-specific silencer. A 13-base pair fragment residing within that region shows 77% sequence identity with the neuron-specific silencer motif recently identified in two neuronal genes, i.e. SCG10 and type II sodium channel genes. We propose that the interplay between the CRE and this neuron-specific silencer region plays an important role in the tissue-specific expression of the DBH gene in noradrenergic cells.

The differential expression of catecholamine biosynthetic enzymes fundamentally distinguishes the biochemical phenotypes of both catecholamine-producing neurons and neurosecretory cells. Thus, dopaminergic neurons express only the first two enzymes in the catecholamine pathway, tyrosine hydroxylase (TH; EC 1.14.16.2.) and L-aromatic amino acid decarboxylase (EC 4.1.1.28.), and thereby synthesize dopamine. Noradrenergic and adrenergic cells express dopamine $\beta$-hydroxylase (DBH; EC 1.1.17.1), enabling them to synthesize norepinephrine. Finally, only adrenergic cells express phenylethanolamine N-methyltransferase (EC 2.1.1.28.), rendering them the only aminergic cells capable of producing epinephrine (Goodall and Kirshner, 1957). Neither the mechanisms governing differential expression of the foregoing enzymes within neurons and neurosecretory cells nor the mechanisms underlying neuron-specific gene expression are well understood. Those overlapping areas of inquiry are among the most important themes in molecular neurobiology. We report here the results of experiments to elucidate molecular mechanisms regulating expression of the DBH gene in the norepinephrine-synthesizing human neuroblastoma SK-N-BE(2)C cell line (Ciccarone et al., 1989), as well as those contributing to suppression of DBH expression in the non-neuronal HeLa cell line.

Recently, two laboratories independently demonstrated that a silencer region is directly involved in neuron-specific expression of genes encoding SCG10, a neuron-specific growth-associated protein (Mori et al., 1990), and the type II sodium channel protein (Maue et al., 1990). 5'-proximal sequences from each of those genes direct transcription in a variety of non-neuronal cell lines, but only after deletion of a putative silencer region. The silencer region thus appeared in large part to determine cell-specific expression of these neuronal genes (Maue et al., 1990; Mori et al., 1990). Surprisingly, further analyses of the silencer function by both groups revealed that the sclencers working in these unrelated neuronal genes shared striking homologies. Moreover, the same sequence-specific poroteins(s), present only in nuclear extracts from non-neuronal cells, bind to both sequences (Kramer et al., 1991; Mori et al., 1992). The foregoing studies suggest that negative genetic elements play a critical role in determining tissue-specific expression of various neuronal genes.

Numerous in vivo and in vitro experiments indicate that the TH and DBH genes share common features of regulation in response to various extracellular stimuli such as nerve growth factor (Acheson et al., 1984; Badoyannis et al., 1991), glucocorticoids (Otten and Thoenen, 1976; Kim et al., 1993c), cAMP analogues (Sabban et al., 1983; Lemounoux et al., 1993), and reserpine (Buguet et al., 1986; Wessel and Joh, 1992). Consistent with these observations, sequence analyses of 5'-flanking regions demonstrated that the TH and DBH genes share various relevant cis-acting motifs, e.g. cAMP-response element (CRE), glucocorticoid response element, AP1, AP2 (Kobayashi et al., 1989; Cambi et al., 1989). In the present report, we isolated a genomic clone containing the 5'-flanking sequence of the human DBH gene and characterized the 4.3-kb upstream region by deletional and mutational analyses using transient expression assay in DBH-positive and DBH-negative human cell lines. We describe the identification of two genetic elements, one positive element and one silencer...
region, required for cell-type specific expression of the human DBH gene.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Human neuroblastoma SK-N-BE(2)C, rat C6 Gliona, and HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated newborn calf serum. Mouse Ltk− cells were grown in Dulbecco’s modified Eagle’s medium:nutrient mixture F-12 (1:1) with 10% heat-inactivated fetal calf serum. All culture media contained 100 units/ml penicillin and 100 µg/ml streptomycin.

**Northern Blot Hybridization**

Poly(A)* RNA was prepared by oligo(dT)-cellulose affinity chromatography (Badley et al., 1988) and was subjected to Northern blot hybridization as described (Kim et al., 1993a). A DNA fragment corresponding to the nucleotide sequence from 1234 to 1666 bp of the human DBH cDNA (Lamouroux et al., 1987) was isolated by polymerase chain reaction, confirmed by sequence analysis, and used as a probe.

**Analysis for DNase I Hypersensitivity Sites**

Nuclear suspensions were prepared as described previously (Iq et al., 1989) with the following modifications. Cells were grown to a concentration of ~5×10⁶ cells/100-mm plate and washed twice with phosphate-buffered saline containing 2.5 mM EDTA. Nuclei were prepared by lysing the cells in 0.5 ml of ice-cold cell lysis buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5% Triton X-100, and 0.25 M sucrose) for 3 min at 4°C. Different concentrations of DNase I (0, 25, 50, 100 units) in 4.5 ml of digestion buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 1 mM CaCl₂, and 0.25 M sucrose) were directly added to each 100-mm dish and incubated at 20°C for 5 min. Each sample was transferred to a centrifuge tube, and the reaction was stopped by adding SDS to 1%, EDTA to 50 mM, and protease K to 200 µg/ml at 37°C for 1 h. DNA was purified by standard methods (Sambrook et al., 1989). 10 µg of puriﬁed genomic DNA (prepared from DNase I-treated nuclei) was digested with EcoRI, separated by 1% agarose gel electrophoresis, and transferred to the nylon transfer membrane using the capillary transfer method for further Southern blot analysis. The filter was prehybridized for 3 h at 42°C in 50% formamide, 5× SSC, 10× polyvinylpyrrolidone, 1% SDS, and 100 µg/ml of sonicated herring sperm DNA. A 0.9-kb HindIII-BamHI fragment encompassing exon 1 of the human DBH gene (Fig. 1) was puriﬁed, labeled by the random priming method to a specific activity of 10⁸ cpmp/µg (Sambrook et al., 1989), and used as a probe. Hybridization was carried out for 1 week at ~80°C with intensifying screen.

**Isolation of the 5'-Upstream Sequence of the Human DBH Gene**

Two oligonucleotides were synthesized and used as primers in polymerase chain reaction as described (Saiki et al., 1988). 5'-GGCTGGGTTGACCTCCCTC-3' and 5'-TCTACTTGGCCGGAGGAGG-3', correspond to locations from -9 to +9 and from -978 to -961 bp of the human DBH upstream region, respectively (Kobayashi et al., 1989). Human genomic DNA prepared from placenta was used as a template. The resulting 987-bp fragment was cloned at the HindII site of pUC19 plasmid and confirmed by sequence analysis. This fragment DNA was then labeled by the random primer method and used as a probe to screen a human genomic library (Clontech). Restriction site mapping and Southern hybridization analyses demonstrated that one of the positive clones, AhuD-2, contained 4.3 kb of the DBH upstream sequence (data not shown). The insert DNA of AhuD-2 was utilized for constructing various CAT-fusion plasmids.

**Plasmid Construction**

**Construction of 4.3CAT and Other CAT-fusion Plasmids Containing Shorter Upstream Sequences of the Human DBH Gene**

A 4.3-kb HindIII-HindIII fragment isolated from AhuD-2 phage DNA was substituted for the corresponding 870-bp HindIII-HindIII fragment of 978CAT plasmid, resulting in 4.3CAT. Orientation of the 4.2-kb insert DNA was confirmed by restriction mapping and sequence analyses. 4.3CAT plasmid contains the upstream sequence of the human DBH gene ranging from -4.3 kb to +9 bp fused to the chloramphenicol acetyltransferase gene. A series of plasmids containing different lengths of upstream sequence were made utilizing the unique SphI site in pBLCAT3 and appropriate restriction sites residing in the upstream region of the human DBH gene (Figs. 1A and 1A).

**Construction of 231CAT, 202CAT, and 189CAT Plasmids**

- To construct plasmids containing upstream sequence between -262 and -175 bp, 978CAT plasmid DNA was digested with PstI and subsequently treated with BamHI enzyme. The plasmid DNA was then digested again with SphI, rendered blunt by Klenow enzyme, and self-ligated. Candidate plasmids were isolated after restriction analysis and confirmed by nucleotide sequence determination.

**Construction of CRE-deleted Mutant Plasmids**

- 978CAT plasmid was partially digested with AatII and treated with T4 DNA polymerase, resulting in ΔCRE978CAT plasmid. Sequence analysis demonstrated that 14 bases between -189 and -176 bp, spanning the CRE, was deleted in this plasmid probably due to nonspecific exonuclease activity of T4 DNA polymerase. ΔCRE978CAT plasmid was digested with PstI and SphI, rendered blunt by Klenow enzyme, and self-ligated to make ΔCRE262CAT plasmid.

**Construction of ΔhDAP604CAT-560CAT**

- ΔhDAP604CAT-560CAT plasmid was digested with Apol and PstI, made blunt-ended, and self-ligated to construct ΔhDAP604CAT plasmid.

**Construction of hDAPCAT-1 and hDAPCAT-2**

- A 224-bp DNA fragment, ranging from -486 to -263 bp of the human DBH gene, was isolated from 468CAT plasmid by complete HindIII and partial Apol digestion. This DNA fragment was placed at the 5'-position of the tk promoter in both orientations, using the HindIII site of pBCAT2 plasmid (Luckow and Schutz, 1987) to produce hDAPCAT-1 and hDAPCAT-2. The orientation of the insert DNA was confirmed by restriction analysis.

**Transient Transfection Experiments**

For transfection analyses, construct plasmids were purified by CsCl gradient ultracentrifugation twice, phenolchloroform (1:1) extraction twice, and ETOH precipitation twice in the presence of 2.5 M ammonium acetate. An equimolar amount of each plasmid construct (0.7 pmol for SK-N-BE(2)C cells and 1.4 pmol for other cells) was introduced into cells by the calcium phosphate co-precipitation method (Gorman et al., 1982a) as described (Kim et al., 1993b). All transfection experiments were repeated two to seven times with similar results utilizing construct plasmids that were independently prepared at least twice. Cells in a 60-mm dish received a total of 5 µg of DNA for SK-N-BE(2)C cell line and 10 µg of DNA for other cell lines. To control for differences in transfection efficiency from dish to dish, 2 µg of pRSV-α-gal plasmid containing the β-galactosidase gene under control of the RSV promoter/enhancer (Edlund et al., 1985) was included in each transfection and used for normalization. PUC19 plasmid was used as a carrier to make up the total amount of DNA. To compare the promoter activities across different cell lines, RSV-CAT plasmids of the equal molar concentration to the CAT fusion construct were introduced into cells in parallel dishes to serve as positive controls (Gorman et al., 1982b; Kramer et al., 1992). 40 h after transfection, cells were extracted and CAT, and β-galactosidase activities were determined as described (Gorman et al., 1982a; An et al., 1982).

**RESULTS**

**DNa I Hypersensitive Sites Reside in the 5' Flanking Region of the DBH Gene in SK-N-BE(2)C Cells, but Not in HeLa Cells**

We chose cell lines suitable to study DBH gene regulation after screening mRNA from several different lines by Northern blot hybridization, using a human DBH cDNA probe. The human neuroblastoma cell line, SK-N-BE(2)C, exhibited robust intrinsic expression of DBH mRNA, whereas HeLa cells showed no detectable signal (Fig. 1B).

When the same blot was hybridized with a cDNA probe for the α-tubulin gene, both lanes produced equivalent signals, indicating similar amounts of mRNA were present in each lane.

We then compared the chromatin structure of the DBH gene promoter in nuclei isolated from SK-N-BE(2)C and HeLa cell lines by treating nuclei with increasing amounts of DNa I prior to DNA isolation. The purified DNA was digested with EcoRI, fractionated by agarose gel electropho-
resis, and subjected to Southern analysis. A 0.9-kb HindIII-BamHI fragment encompassing exon 1 served as a probe in the Southern blot and an EcoRI site at +1.35 kb position in the first intron served as a reference position (Fig. 1A). The 5'-proximal promoter region of the human DBH gene revealed a cell type-specific pattern of DNase I hypersensitive sites (HSSs). Two prominent DNase I HSSs appeared at about 1.35 and 1.5 kb upstream of the reference EcoRI site in the nuclei isolated from SK-N-BE(2)C cells (Fig. 1, A and C). These sites mapped to proximal promoter regions residing approximately at -30 to -20 bp and -190 to -170 bp, corresponding respectively to the TATA box (-29 to -24; ATAAAAT) and CRE (-181 to -174; TGAGCGTCC) (Fig. 1D). In addition, there was another DNase I HSS with weaker signal at 2.5 kb upstream of the EcoRI site. None of these DNase I HSSs were detected in nuclei from HeLa cells, suggesting that the proximal promoter region of the human DBH gene maintains an open chromatin structure accessible to DNase I only in DBH-expressing cells. We hypothesized that the three DNase I HSSs represented active promoter elements involved in cell type-specific expression of the human DBH gene. To test this hypothesis, we performed mutational analyses using SK-N-BE(2)C and HeLa cell lines, as models exhibiting cell type-specific expression or repression of the DBH gene, as follows. 4.3- and 2.6-kb Upstream Sequence of the Human DBH Gene Direct the Expression of the Reporter Gene in a Cell Type-specific Manner—After isolating and partially characterizing a genomic clone containing >4.3-kb upstream region (see “Experimental Procedures”), we used HindIII and SacII sites (Fig. 1A) to fuse the upstream 4.3- and 2.6-kb sequence 5' to the reporter gene encoding CAT in the promoterless plasmid, pBLCAT3 (Luckow and Schütz, 1987). Fusion was made at position +9 in the untranslated sequence of the human DBH mRNA (Fig. 2A). The resultant constructs, 4.3CAT and 2.6CAT, also contain intron and polyadenylation signal in the 3'-nontranslated region which originated from SV40 (Luckow and Schütz, 1987). When introduced into
Deletions from -604 to -486, and to -262 bp, did not produce any significant change in CAT activity. However, further deletion of 87 base pairs up to -175 bp or to -114 bp virtually eliminated CAT activity, rendering it transcriptionally as inert as pBLCAT3 (Fig. 3B). These data suggest that essential positive element(s) reside in the upstream region between -262 to -175 bp, a region that contains one of the strong DNase I HSS identified above (Fig. 1C). To define further the sequence motif of the positive genetic element, we produced smaller deletions within this region using Bal31 endonuclease digestion (Fig. 4A). All three deletional constructs between -262 and -175 bp, i.e. 262CAT, 202CAT, and 189CAT, retained transcriptional activity similar to that of 262CAT, indicating that an essential positive element resides in the region between -189 and -175bp (Fig. 4B). This area contains a potential cAMP response element, -181 TGACGTCC -174 (Fig. 1D), with a single base deviation from the consensus CRE sequence (TGACGTCA; Montminy et al., 1986). We thus performed site-directed mutagenesis of this putative CRE in the intact upstream sequence to test if (i) the CRE is an essential genetic element for the expression of the human DBH gene and (ii) this CRE motif is functional in response to elevated levels of cAMP. CRE978CAT, in which the 14 base pairs between -189 and -176 bp are deleted in the context of 978 bp of intact upstream sequence, reduced expression of 978CAT plasmid by >95% (Fig. 4C). Moreover, the response of CRE978CAT plasmid to treatment with dibutyryl cAMP was decreased compared to that of 978CAT (Fig. 4C). We concluded that the CRE residing between -189 and -174 bp is a functional CRE. These data define the CRE, residing at -181 to -174 bp upstream of the transcription initiation site, as an essential positive element in the expression and regulation of the human DBH gene.

We also performed parallel transient expression assays in HeLa cells in an attempt to identify any regulatory element involved in the cell type-specific transcriptional suppression of the DBH gene in this cell line. None of the fusion constructs containing the promoter sequences deleted between -4.3 kb and -486 bp showed CAT activity higher than that directed by pBLCAT3 (Fig. 3C), indicating that the 486-bp upstream sequence contains sufficient information to suppress expression in HeLa cells. Further deletion to -262 bp, however, resulted in derepression of the CAT activity approximately by 10-fold compared with that by 486 bp upstream (Fig. 3C). Thus, the upstream region between -486 and -262 bp appears to contain one or more negative elements that are required for effective suppression of the DBH gene in HeLa cell line. The derepressed promoter activity of 262CAT plasmid in HeLa cells relative to the RSV promoter was approximately 10% of 262CAT activity in SK-N-BE(2)C cells (Fig. 3, B and C). To further characterize the negative region, we deleted the sequence between -490 and -262 bp in the 604CAT plasmid (Fig. 5A) and compared the promoter activity of the resulting construct in both cell lines (Fig. 5B). As expected, deletion of this region in the context of 604-bp upstream sequence did not alter promoter activity in SK-N-BE(2)C cell line. In sharp contrast, however, this deletion caused about 10-fold derepression of CAT activity in HeLa cells (Fig. 5B) as well as in mouse Ltk" cells (data not shown), confirming that this region contains a cell type-specific repressing sequence. Interestingly, the fusion constructs lost transcriptional activity upon deletion to -175 bp or to -114 bp, indicating that the upstream region between -262 and -114 bp contains positive element(s) required for the derepressed
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Fig. 3. A, schematic diagram illustrating deletion constructs of human DBH-CAT fusion gene. Restriction enzyme sites used to construct the DBH-CAT fusion gene are shown at the top. The transcription initiation site is indicated by the bent arrow. The 5'-end point of each construct is shown at the left of each bar. B and C, analysis of CAT activities in human neuroblastoma SK-N-BE(2)C cells (B) and HeLa cells (C) by transient expression assays. The percent conversion of chloramphenicol to butyrylated chloramphenicol is indicated at the right of each autogram. This experiment was performed seven times in triplicate with similar results. A representative result is shown here. pRSV-β-gal was included in transfection as an internal control to normalize variation of transfection efficiencies between different DNA precipitates. Cell extracts corresponding to the same β-galactosidase activity were used in the CAT assay. Since pRSV-CAT had very high CAT activity, a much smaller amount, 1/10 compared with other fusion plasmid, was used for measurement of the CAT activity.

Fig. 4. Identification of the CRE region as an essential regulatory element. A, schematic diagram illustrating detailed deletion constructs and a CRE-deleted construct. B, location of an essential positive element by detailed deletion analysis. These constructs were prepared using Bal31 exonuclease as described under "Experimental Procedures." C, site-directed mutagenesis of the CRE region. Fourteen bases spanning the CRE were in ΔCRE978CAT. Sixteen hours prior to harvest, cells were treated with dibutyryl cyclic AMP to assess the functionality of this putative CRE motif (as illustrated by + in parentheses next to each plasmid construct). ΔCRE978CAT construct lost not only the basal expression but most of its responsiveness to treatment of dibutyryl cyclic AMP. This experiment was performed twice in triplicate with similar results.
Upstream of Human DBH Gene, Contains Two Sequence Patches Highly Homologous to Previously Identified Neuron-specific Sequence Motifs—Our analyses of the 4.3-kb upstream region of the human DBH gene revealed a 223-bp fragment which conferred a repressing effect to the native DBH promoter in a cell type-specific manner. We determined whether this region of the human DBH gene contained sequences similar to previously described neuron-specific sequence elements by comparing the nucleotide sequences. These searches revealed two sequence patches sharing significant homologies with such sequence motifs previously identified in other neuronal genes (Fig. 5C). First, the sequence between −456 and −444 bp shared 77% identity with a cis-acting element initially described in the Drosophila Dopa decarboxylase gene. This element, designated element I, was protected in DNase I footprints by embryonic nuclear extract and was necessary but not sufficient for neuron-specific expression in the Drosophila central nervous system (Scholnick et al., 1986; Bray et al., 1988).

Another sequence was found at −400 and −384 bp, which is homologous to the silencer sequence motifs independently identified in two different mammalian neuron-specific genes, i.e. rat type II sodium channel and SCG10 genes (Kraner et al., 1992; Mori et al., 1992; Fig. 5C). This silencer sequence predominantly determines the neuron-specific expression of these genes via a presumably identical silencer-binding protein(s). The 21 nucleotide sequences, which retained silencer function by themselves in type II sodium channel and SCG 10 genes, shared 81% sequence identity with each other (Kraner et al., 1992; Mori et al., 1992). The sequence found in the human DBH gene shows less homology with these motifs: 48% with SCG10 and 57% with type II sodium channel gene sequences (Fig. 5C). However, a subdomain of 13 nucleotides (−400 to −388 bp) within this putative silencer region of the human DBH gene shared 77% identity to the corresponding fragments from the SCG10 gene or from the type II sodium channel gene.

The Negative Element Region Exerts the Silencing Effect on a Heterologous Promoter in a Cell Type-specific Manner—To assess whether the foregoing putative silencer region can confer negative regulation upon a heterologous promoter independent of orientation, as would be expected if it functions analogously to the silencer present in SCG10 and type II sodium channel genes (Mori et al., 1992; Kraner et al., 1992), we placed this 223-bp fragment of putative silencer region in front of the thymidine kinase (tk) promoter using pBLCAT2 in either orientation (Fig. 6A, Luckow and Schutz, 1987). When introduced into SK-N-Be(2)C cells, the properly oriented fragment did not inhibit transcriptional activity of the tk promoter; however, in the opposite orientation, this fragment inhibited tk promoter-supported CAT activity by 50% compared with the wild-type tk promoter. In contrast, in HeLa cells, this sequence suppressed tk-supported CAT activity to less than 50% of control in either orientation (Fig. 6B).
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Thus, the region between −486 and −263 bp of the human DBH gene appears to contain an element that acts as a silencer in non-neural cells, but not in neural cells. The suppression effect of this sequence was more effective when linked to its native promoter than when attached to a heterologous tk promoter (compare Fig. 3, B and C, with Fig. 6B).

DISCUSSION

The transcriptional regulation of many eukaryotic genes has been intensively studied during the last two decades, and tissue-specific enhancers for different cell types, e.g. liver (Cereghini et al., 1987), lymphoid cells (Grosschedl and Baltimore, 1985), pancreas (Cockell et al., 1989), and anterior pituitary (Lefevre et al., 1987) are well established. Nevertheless, the molecular mechanisms underlying neuron-specific gene expression are poorly understood. In the present study, we examined the cell type-specific expression of the human DBH gene by comparing its promoter function in a human noradrenergic cell line to that in non-neuronal non-catecholaminergic cell lines.

As a first step in identifying important genetic elements involved in the cell type-specific DBH expression, we tested whether the 5′-flanking sequence of the human DBH gene contained cell type-specific nuclease-sensitive sites. DNase I HSS mapping analysis revealed three sites in the 5′-proximal sequence in SK-N-BE(2)C cells but not in HeLa cells (Fig. 1C). The two proximal sites of these DNase I HSS, located at about −25 and −180 bp, respectively, corresponded to the locations of the TATA box and CRE. Since active promoter elements often exist as nuclease-sensitive structures in chromatin, our data indirectly suggest that the proximal area of the human DBH gene contains several cis-acting elements which may be important for cell type-specific expression. Thus, we next sought to address whether the upstream sequence of the human DBH gene can direct expression in a cell-specific manner. When transiently expressed in different cell lines, both the 4.3- and 2.6-kb upstream sequences did not affect the expression of the reporter gene in a tissue-specific manner (Fig. 2), consistent with previous results obtained in transgenic mice (Mercer et al., 1991), demonstrating that the 5.8-kb upstream sequence of the human DBH gene can direct tissue-specific expression. Our data further suggest that the sufficient information for cell type-specific expression may be confined to the 2.6-kb upstream region.

The 2.6-kb upstream sequence was further examined using extensive deletional and mutational analyses. Transient expression assays in SK-N-BE(2)C cells revealed that the 5′-flanking sequence of the DBH gene may contain several regulatory cis-acting elements. First, upon deletion of the sequence from −1556 bp to −978, and to −604 bp nucleotide position, CAT activity progressively increased about 2-fold (Fig. 3B), suggesting that this region might contain negatively acting element(s) active in the SK-N-BE(2)C cell line. Second, and more importantly, the nucleotide sequences between −189 to −176 bp appeared to contain a critical positive element supporting transcriptional promoter activity of the human DBH upstream sequence (Fig. 4). This region contains a consensus CRE with a single base deviation (A to C at −174 bp position; Fig. 1D). Deletion of the 14 bp region (from −189 to −176), including the CRE, in the context of 978 bp of intact upstream sequence, not only caused a severe decrement in the basal promoter activity but also reduced its responsiveness to the elevated cAMP (Fig. 4C). In contrast, the CAT activity driven by the wild-type promoter, 978CAT, increased about 2.5-fold upon treatment with dibutyryl cAMP. These data strongly suggest that the CRE, residing at −181 to −174 bp, plays an important dual role in the DBH promoter: (i) it supports the uninduced, basal expression of DBH and (ii) mediates, at least in part, cAMP-inducible expression in noradrenergic cells.

While this paper was being prepared, two laboratories reported the results of analyzing the 5′-promoter region of the DBH gene. First, Shaskus et al. (1992) examined the 395-bp upstream sequence of the rat DBH gene and showed that a 30-bp region between −180 and −151 bp might contain a transcriptional enhancer which had a dual function for cell type specificity and second messenger responsiveness. Second, Lamouroux et al. (1993) analyzed the 1247-bp upstream sequence of the human DBH gene and showed that the upstream region between −267 and −115 bp was crucial for cell-specific and cAMP regulation, suggesting the importance of a near-consensus CRE located in this region. These data generally agree well with ours. On close inspection of the nucleotide sequences in these overlapping promoter regions; however, it became evident that the human and rat genes contain different compositions of nucleotide sequences in putative cis-acting elements in this area (Fig. 1D). The rat gene contains a sequence motif, TGCTGCA, which is a canonical AP1-like motif (Shaskus et al., 1992). The corresponding position of the human gene has a similar motif, TGTGTCGA, with a single base variation (Fig. 1D). As both 175CAT and ΔCRE978CAT retain this sequence and do not show CAT activity any greater than pBLCAT3 (Fig. 4), it appears that this motif, TGTGTCGA, in and of itself, does not exert transcriptional activity. We suggest here that the CRE with a single base deviation, which in the human gene is just proximal to this AP1-like motif, is functionally essential for transcriptional activity. The corresponding rat sequence contains a CRE-like motif which varies by 2 bases from the consensus CRE motif (Fig. 1D). In contrast to the functional importance of the CRE in the human gene, transient expression analysis by Shaskus et al. (1992) suggests that the CRE-like motif is functionally silent in the rat DBH gene. Clear delineation of
the functional importance of these sequence motifs in regulation of the human and the rat DBH genes awaits further investigation.

Both of the rat and human TH genes contain the consensus CRE in the proximal region (Kobayashi et al., 1988; Cambi et al., 1989). Recent data from our laboratory suggest that the CRE plays an important dual role in both basal and cAMP-inducible expression in the TH gene (Kim et al., 1993b). Thus, it appears that two enzyme genes of the catecholamine pathway, TH and DBH, adopt similar molecular strategies by utilizing the same cis-acting element (CRE) for their expression and regulation. The CRE has been inferred to be important for the basal expression of several other genes (Deutsch et al., 1987; Delegueane et al., 1987; Quinn et al., 1988; Andrisani et al., 1989). It is plausible that a cAMP-dependent protein kinase signaling pathway might be directly involved in regulating the expression of these genes. Indeed, we recently demonstrated that the CAMP-dependent protein kinase system regulates both the basal and CAMP-inducible expression for the TH gene (Kim et al., 1993a) as well as the DBH gene.

Recent studies have identified a number of different genes where silencers play important roles in transcriptional regulation (Renkawitz, 1980). Our transient expression analyses indicate that an upstream region of the human DBH gene might play an important role for the cell type-specific expression of this gene. The promoter activity, however, reached only 10%, upon deletion of the putative silencer region, in HeLa cells compared with SK-N-Be(2)C cells (Fig. 3). This contrasts with the SCG10 and type II sodium channel genes where the proximal sequence devoid of the silencer displayed similar promoter strength in neuronal and non-neuronal cells (Mori et al., 1992; Kraemer et al., 1992). These data suggest that the proximal 262 bp of the DBH promoter contributes to cell type-specific expression as indicated by other investigators (Shakusk et al., 1992; Lamouroux et al., 1993). The CRE appears to be an essential positive element which is required for transcriptional activity by the upstream sequence of the human DBH gene. Consequently, the corresponding CRE-binding proteins appear to play a central role in transcriptional activation of the human DBH gene in noradrenergic and adrenergic cells. In DBH-nonexpressing cells, the action of such transcription factors, if present, should be neutralized by the silencer residing farther upstream, thus rendering the DBH gene transcriptionally silent. Our observation, that the 262CAT plasmid which lacks the silencer region loses the derepressed expression in HeLa cells upon deletion of the CRE (Fig. 5), supports that contention. Thus, in the absence of the silencer region, derepressed expression by the upstream sequence of the human DBH gene requires a functional CRE. These data suggest that the interplay between the CRE-binding protein(s) and silencer-binding protein might represent, at least in part, an underlying mechanism for tissue-specific expression of the human DBH gene.

A clearer understanding of the foregoing mechanisms will be possible once the proteins that bind to the CRE and the silencer motif of the human DBH gene have been identified, cloned, and characterized. That would also make it possible to test whether the same silencer-binding protein or a family of similar proteins is involved in determining both neuron-specific expression and neuronal subtype-specific distribution of a variety of genes.

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