Dopamine β-hydroxylase, the enzyme which converts dopamine to norepinephrine, is expressed in a cell type-restricted pattern in neuroendocrine tissue. A segment of the rat gene containing 395 bases of 5'-flanking sequence regulates expression of a reporter gene in a cell type-selective pattern in mammalian cell cultures. Using deletion mutants of the 5'-flanking sequence, we have identified a 30-base genetic regulatory element, designated DB1, which enhances transcription from a heterologous promoter 5–20-fold in neuroendocrine cell lines. DB1-specific DNA-protein complexes are found in nuclear extracts from all cell lines examined, but the migration pattern differs between cell lines.

The 5'-flanking region of the dopamine β-hydroxylase gene is also responsive to cyclic AMP and phorbol ester treatment of SHSY-5Y neuroblastoma cells. The simultaneous presence of both effectors results in synergistic increases in DBH mRNA and reporter gene activity. The second messenger regulatory element was localized to the region containing the DB1 element, and reporter plasmids containing multiple copies of the DB1 element are responsive to treatment with inducers. The results of this study identify a cis-acting regulatory element which influences both cell type selectivity and second messenger responsiveness of the rat dopamine β-hydroxylase gene.

The functional specificity of a tissue is largely determined from the repertoire of genes transcribed in that tissue. Cells of neuroendocrine tissue contain gene products that are common to many functionally distinct cell types, such as those proteins involved in stimulus-coupled secretion. In addition, each specialized group of neuroendocrine cells expresses gene products unique to the function of these cells. Proteins in this group include ion channels, neurotransmitter or hormone receptors, and neurohormone biosynthetic enzymes. Expression of the appropriate neuroendocrine phenotype can be modulated by developmental, spatial, and local environmental influences (LeDouarin, 1990; Landis, 1990; He and Rosenfeld, 1991).

The biosynthesis of the neurotransmitter norepinephrine is catalyzed by dopamine β-hydroxylase. This enzyme is expressed in specified brain nuclei such as the locus ceruleus, in the sympathetic ganglia, and in the endocrine tissue of the adrenal medulla. In adult animals, all tissues that express dopamine β-hydroxylase also express the preceding enzymes of the catecholamine pathway, tyrosine hydroxylase and dopa decarboxylase. Thus, the presence of dopamine β-hydroxylase in a tissue will usually determine that the tissue will synthesize norepinephrine and will become either noradrenergic or adrenergic.

In addition to a cell type-restricted expression of dopamine β-hydroxylase, the enzyme activity and mRNA levels are altered in response to environmental stimuli, such as stress (Kvetnansky et al., 1971; McMahon et al., 1992). The intracellular signals mediating the response to extracellular stimuli may involve known second messenger pathways, such as cyclic AMP-dependent protein kinase or protein kinase C. Several other genes expressed in neuroendocrine tissue, including those encoding tyrosine hydroxylase (Lewis et al., 1987; Vysocki et al., 1989), proenkephalin (Comb et al., 1986, 1988), and vasoactive intestinal peptide (Fink et al., 1988, 1991) are responsive to stimulation by both of these pathways.

The cell type-specific expression of many genes has been shown to be influenced by genetic regulatory elements present in the 5'-flanking region of those genes. In some instances, a transcriptional modulator unique to that tissue will bind cis-acting regulatory sequences that specify the expression of genes required for specialized function (Weintraub et al., 1991; Ingraham et al., 1988). In other instances cell type-specific transcriptional activation is influenced by transcriptional factors, which also function to regulate expression of genes in response to second messengers (Powers et al., 1989; Bokar et al., 1989; Boshart et al., 1991; Jones et al., 1991). Many genes are under the control of both positive and negative regulatory
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Factors, and the restricted expression of the dopamine $\beta$-hydroxylase gene suggests that multiple genetic elements may be involved in transcriptional modulation.

In this study we investigated several aspects of dopamine $\beta$-hydroxylase gene expression, including: (1) a molecular analysis of genetic regulatory elements which influence basal and cell type-selective expression and (2) the response of the gene to stimulation of cyclic AMP and phospholipase C second messenger pathways. In order to understand the factors which influence the expression of this gene we have isolated a genomic clone containing rat dopamine $\beta$-hydroxylase DNA. In this report, we describe our studies characterizing the structural and functional organization of a segment of the gene containing 395 bases 5'-flanking the start site of initiation. The results of our experiments identify a genetic regulatory element involved in basal, cell type-selective, and second messenger responsiveness of dopamine $\beta$-hydroxylase gene expression.

**EXPERIMENTAL PROCEDURES**

Cell Culture—All cell lines were cultured in a humidified atmosphere containing 5% CO$_2$ at 37 °C. SH-SY5Y human neuroblastoma were cultured in 1:1 F-12/MEM plus 10% fetal calf serum, CV-1 monkey kidney and JEG-3 chorioncarcinoma cells were maintained in Dulbecco's MEM supplemented with 10% fetal calf serum, while C6 glioma cells were cultured in Dulbecco's MEM supplemented with 5% fetal calf serum. PC12 cells were maintained in RPMI plus 10% horse and 5% fetal calf serum. All sera were purchased from HyClone Laboratories (Logan, UT).

Screening of $\lambda$ Phage Libraries—Phage from a rat EMBL-3 library were screened with a cDNA probe of bovine dopamine $\beta$-hydroxylase containing 1.2 kilobases of the 3'-terminal sequence (Lewis et al., 1990), which had been radiolabeled with $^{32}$P by nick translation. Conditions for hybridization were as previously described (Lewis et al., 1990). Following plaque purification of positive clones, $\lambda$ DNA was purified using Ambis Laboratories (Promega Biotech) and the protocol provided by the manufacturer.

DNA Sequencing—Fragments of the dopamine $\beta$-hydroxylase gene which were to be sequenced were subcloned into the plasmid pGem3Z (Promega Biotech) and DNA sequence analysis was performed on double-stranded DNA using the dideoxy chain termination method with Taq polymerase and the protocol described in the TaqTrack provided by the manufacturer.

DNA Sequencing was performed using the dideoxy chain termination method described in the TaqTrack provided by the manufacturer.

PCR was performed using the dideoxy chain termination method described in the TaqTrack provided by the manufacturer.

**Northern Blot Analyses**—Total cellular RNA was extracted from SH-SY5Y cultures using the LiCl/urea precipitation method described above. 10 μg of total RNA extracted from these cultures was fractionated on formaldehyde-agarose gels as described in Sambrook et al. (1989). Blots were probed with a 0.7-kilobase probe derived from exon 12 of the human DBH gene (Kobayashi et al., 1989), which was radiolabeled by nick translation. Following hybridization and autoradiography, the intensity of the hybridization signal was quantitated by use of an Ambis Imaging System.

Transfection of DNA into Cultured Cells—DNA for transfection was isolated from the alkaline lysate using the alkaline lysis method (Birnboim and Doly, 1979) followed by precipitation with polyethylene glycol. The DNA-containing pellet from the alkaline lysis procedure was resuspended in H$_2$O. Sodium chloride was added to a final concentration of 0.4 M, and an equal volume of 13% polyethylene glycol 8000 was then added. Samples were incubated at 0 °C for 30-60 min, at which time the plasmid DNA was pelleted by centrifugation at 100,000 × g. The pellet was resuspended in water, extracted twice with chloroform, and precipitated with alcohol.

Transfection of plasmid DNA into cultured cells was performed using the calcium phosphate method originally described by van der Eb (Graham and van der Eb, 1973). In experiments where the expression of reporter gene activity was to be compared between different cell lines or different plasmid constructs, a standardization plasmid containing a luciferase transcription unit under the control of promoters from either Rous sarcoma virus (RSV-Luc; de Wet et al., 1987) or herpes simplex virus thymidine kinase (TK-Luc; Gorman et al., 1988) were included in the transfection. Cultures were harvested two days after transfection. Cells were extracted in 50-100 μl of 0.1 M potassium phosphate, pH 7.8, plus 1 mM dithiothreitol. One to five μg of cell extract were used for assay of luciferase activity (deWet et al., 1987), whereas 30-70 μl were used for assay of chloramphenicol acetyltransferase activity (Usman et al., 1987). The luciferase assay was performed on a Packard Picolite luminometer, and the CAT assay was performed using thin layer chromatography.

Construction of Recombinant Clones—All procedures in the construction of recombinant clones utilized standard molecular biology protocols, described in Sambrook et al. (1989).

For construction of the recombinant DNAs containing a segment of the dopamine $\beta$-hydroxylase gene adjacent to the CAT transcription unit, a SalI/SalI restriction fragment of the original $\lambda$ clone containing dopamine $\beta$-hydroxylase sequences was subcloned into vector pEGM 3Z. A HindIII site from the vector was used in conjunction with the Fnu4HI site, which digests +14, to produce a 499-base fragment which was cloned into the HindIII/Smal sites of pUC CAT (Lewis et al., 1987). This segment contains the sequence ATG, a potential translational initiation codon, beginning at +10. Utilization of this ATG codon in the mRNA transcript derived from 5'-DBH-CAT would result in a nonsense polypeptide. To prevent utilization of this AUG in the mRNA, the ATG at +10 of 5'-DBH-CAT was mutated to AGG, a codon which is a very poor translational initiator (Peabody, 1989). The 5'-DBH-CAT plasmids utilized in the experiments reported in this study contain this mutation.

Recombinants which contain progressive deletions of the dopamine $\beta$-hydroxylase 5'-flanking sequences from the 5'-DBH-CAT construct were made using the pDBH-CAT construct described above. The 5'-DBH-CAT (−395/+14 ) plasmid was digested with SpI1 and SalI, followed by digestion with EcoRI at 34 °C. Aliquots were removed at 15-30 intervals, and digestion was stopped by immersing the samples in reaction buffer for S1 nuclease on ice. After removal of all aliquots, the S1 reaction proceeded at room temperature for 30 min. The ends were made flush by treatment with the Klenow fragment of DNA polymerase in the presence of deoxyribonucleoside triphosphates, and plasmids were ligated with T4 DNA ligase. The ligated plasmids were transformed into JM101 bacteria and individual colonies were screened by restriction digestion patterns and DNA sequence analysis.

Recombinants which contain the 30 base DB1 oligonucleotide segment cloned adjacent to the thymidine kinase (TK) promoter were
made by use of the pTE1 plasmid, whereby CAT transcription is under the control of the TK promoter (Edlund et al., 1985). Oligonucleotides corresponding to the nucleotides -180/-151 of the dopamine β-hydroxylase 5'-flanking region and including a GATC at the 5'-end were synthesized in both orientations. The double-stranded DNA was cloned into the BgII site in the polylinker of pTE1. The 588-base NRL/Afl fragment of pBR322, which separates the nucleotides corresponding to the nucleotides -190/-151 of the dopamine β-hydroxylase gene, was then removed so that the DB1 segment was directly adjacent to the TK promoter. Recombinants were verified by restriction mapping and DNA sequence analysis.

Site-directed Mutagenesis—The ATG at position +10 of 5'-DBH-CAT was changed to AGG using the Altered Sites in Vitro Mutagenesis System (Promega Biotec). The primer for the mutagenesis protocol, 5'-GCTCCCTGCCTGGCTGGGA-3', contained the reverse complement of the sequence of the dopamine β-hydroxylase gene/CAT hybrid from +2 to +20, with the A corresponding to the +11 position changed to a C. This would change the ATG sequence beginning at +10 in the coding strand to AGG. A 2-kilobase BamHI fragment from the 5'-DBH-CAT (-395/+14) construct was subcloned into the pSELECT-1 vector, from which single-stranded phage DNA was directly adjacent to the TK promoter. Recombinants were generated. The single-stranded DNA was annealed to the mutant primer, along with a second primer designed by the manufacturers, by heating the DNAs at 70 °C for 15 min, followed by cooling to room temperature. The second strand synthesis was performed using T4 DNA polymerase, followed by ligation with T4 DNA ligase. DNAs were transformed into bacteria BMH 71-18 mut S, provided by the manufacturer, and colonies were screened by antibiotic resistance and DNA sequence analysis.

Preparation of Nuclear Extracts—Nuclear extracts from tissue culture cells were prepared by the method of Dignam et al. (1983).

Electrophoretic Mobility Shift Assay—The interaction of the 5'-flanking region of dopamine β-hydroxylase DNA with nuclear factors was analyzed using the electrophoretic mobility shift assay (EMSA). For assays where the cDNA for rat somatostatin gene, 5'- CGTGGGTGAGCGGGGAGAGAGAGAGAG-3', and for the rat dopamine β-hydroxylase DB1 segment were synthesized in house.

RESULTS

Isolation of the 5'-Flanking Sequences of the Rat Dopamine β-Hydroxylase Gene—In order to isolate cloned DNAs containing the rat dopamine β-hydroxylase gene, a rat genomic library cloned in λ EMBL 3 was screened with a cDNA for bovine dopamine β-hydroxylase (Lewis et al., 1990). Three identical clones were isolated from an initial screen of 2.5 × 10⁶ plaques. In order to localize the region surrounding the initiation site of transcription, Southern blot analysis was performed using a degenerate oligonucleotide corresponding to bases +96 to +115 in the 5'-proximal region of the human dopamine β-hydroxylase cDNA (Lamoureux et al., 1987). Extensive restriction analysis identified a 600-base SalI/SfiI fragment which hybridized to this oligonucleotide. The SalI site is derived from the vector and represents the 5'-boundary of the inserted DNA in all three clones. The 600-base fragment was subcloned into pGEM 3Z (Promega) and subjected to T-dNA sequence analysis (Fig. 1A).

A comparison of the sequence of the 600-base rat genomic DNA to that of the human cDNA and gene (Lamoureux et al., 1987; Kobayashi et al., 1989), as well as the cDNA for rat dopamine β-hydroxylase (McMahon et al., 1989), suggests that this fragment contains 395 bases of sequence 5' to the initiation site of transcription and 200 bases of transcribed sequence. The transcribed DNA is identical to that previously reported for the rat cDNA (McMahon et al., 1990), and is not interrupted by an intron. Twenty-eight bases upstream from the designated 5'-terminus of the rat transcription unit is the sequence ATATAAT, which is likely to function as the promoter recognition site for RNA polymerase II. Several regions of the rat dopamine β-hydroxylase gene contain sequence homology to motifs demonstrated to influence gene expression in other systems. Two elements are present which are similar to the consensus binding sequence for AP1 (TGA(C/G)TCA),
the transcriptional modulatory complex composed of members of the fos and jun gene families (see Mitchell and Tjian (1989) and Sheng and Greenberg (1990)), beginning at positions -367 and -173. Sequence identical to the ENK-CRE2 cyclic AMP/phorbol ester response element of the proenkephalin gene (Comb et al., 1986) is found beginning at site -173. Two segments similar to the consensus for AP2 (GCCCGAGGC) (see Mitchell and Tjian (1989)), a transcriptional activator which has been shown to modulate the extent of the transcriptional response from the ENK-CRE2 element of the proenkephalin gene (Hyman et al., 1989), are found at positions -129 and in the reverse orientation at -6.

The 5'-flanking regions of the rat and human dopamine β-hydroxylase genes were compared to identify segments which are conserved between genes and could potentially correspond to genetic regulatory elements which function in the modulation of dopamine β-hydroxylase transcription. When sequences are aligned for maximum homology, the 395 bases of the promoter proximal region (Fig. 1B). Two segments that were identified as potential regulatory elements in the human gene (Kobayashi et al., 1989) are not identical in the rat. These are: 1) the CCAAT motif beginning at -159 in the human, which is changed to TCAAT in the rat gene, and 2) the sequence TGACGTCC, found at -181 in the human gene and identified as a potential cyclic AMP response element, which is changed to TGAATGTCC in the rat gene, beginning at -183. However, as described above, at position -173 there is a sequence element similar to the cyclic AMP response element of the human proenkephalin gene. It is possible that both genes possess a cyclic AMP response element, which is conserved in function but not in absolute sequence and location. In addition to those elements described in the above paragraph, there are several more regions of similarity with unknown functional significance. The role of these potential regulatory elements will be examined using tissue culture and in vitro assays.

**Dopamine β-Hydroxylase RNA Transcripts Contain the Same 5'-Terminus in Several Tissues**—The 5'-terminus of the dopamine β-hydroxylase RNA transcript was ascertained in several tissues, using an RNase protection assay. An antisense RNA transcript was prepared from the Stl(+200) to EcoRII(-87) sites of the genomic clone and was hybridized to RNA extracted from rat tissues which express dopamine β-hydroxylase, including the adrenal gland, the brain nucleus locus ceruleus, and the sympathetic superior cervical ganglia, as well as to RNA from tissue which does not express the protein. The RNA from all three dopamine β-hydroxylase-expressing tissues produced a nuclease-protected band (Fig. 2) that corresponds in size to the transcript expected if transcription initiation at the site predicted by DNA sequence analysis (Fig. 1A). RNA extracted from rat liver and kidney produced no band, while RNA from the rat pheochromocytoma cell line PC12 produced a faint band which co-migrated with that of neural tissue. The PC12 cell line originally produced dopamine β-hydroxylase (Greene and Tischler, 1976), but has since diminished expression. The same low level of expression was observed in RNA extracted from subclones of PC12 derived from three different sources (data not shown), and no dopamine β-hydroxylase could be detected in these cells by Western blot or enzyme assay (Lewis and Asnani, 1992). The results of the RNase protection analysis demonstrate that the dopamine β-hydroxylase RNA exhibits a restricted tissue distribution and that the 5'-end of the mature RNA transcript is identical in the brain, sympathetic ganglia, adrenal medulla, and pheochromocytoma.

**The 5'-Flanking Sequences Direct Cell Type-specific Reporter Gene Expression**—In order to assess the ability of this putative promoter region to direct transcription in mammalian cells, a DNA construct was developed whereby a segment of the dopamine β-hydroxylase gene corresponding to sequences -395 to +14 was cloned adjacent to the bacterial chloramphenicol acetyltransferase (CAT) transcription unit (Gorman et al., 1982). This hybrid DNA, carried in a pUC-derived plasmid, was transfected into several different cell lines in order to ascertain the ability of different cell types to activate transcription from the dopamine β-hydroxylase promoter. The efficiency of transfection was monitored by cotransfection of the plasmid TK-L, which contains the luciferase transcription unit under the control of the promoter of the thymidine kinase gene from herpes simplex virus (Nordeen, 1988), followed by assay of luciferase in cell extracts. The relative level of CAT activity following transfection of the 5'-DBH-CAT construct into several cell lines was found to be at least 40-fold greater in the SHSY-5Y human neuroblastoma cell line (Fig. 3), which exhibits dopamine β-hydroxylase activity (Ross et al., 1983), than in cell lines which do not express dopamine β-hydroxylase, including CV-1 monkey kidney, JEG-3 choriocarcinoma, or C6 glioma cells (Fig. 3). Expression of 5'-DBH-CAT was also reduced, relative to the neuroblastoma cell line, when transfected into the GH4 rat anterior pituitary cell line, the SH-EP cell line, an epithelial-like cell line derived from the same initial neuroblastoma as SHSY-5Y (Ciccarone et al., 1989), or HeLa cells (data not shown). The results of these experiments demonstrate that there are genetic regulatory elements present in this segment of DNA from the dopamine β-hydroxylase gene which contribute to the specificity of expression of dopamine β-hydroxylase.

**Identification of Sequences in the 5'-Flanking Region of the Dopamine β-Hydroxylase Gene Which Influence Expression**—The 5'-flanking sequences contained in the 5'-DBH-CAT constructs were progressively deleted in order to identify...
regions that contribute to the expression of the gene in the neuroblastoma cells. A series of deletion mutants were constructed from the parental 5'-DBH-CAT (−395/+14) and cotransfected into SHSY-5Y cultures with the RSV-L plasmid used as a transfection standard. In RSV-L, the luciferase transcription unit is under control of the promoter from the Rous sarcoma virus. The level of reporter gene expression derived from transcriptions of the 5'-DBH-CAT plasmids varies little when 5'-flanking sequences from −395 to −232 are progressively deleted (Fig. 4). Deletion of the subsequent 43 bases, to −189, results in a modest decrease in promoter activity, whereas a further deletion of the following 27 bases, from −189 to −163, results in an additional 5-fold diminution in reporter gene activity. Further deletion to −131 reveals an increase in activity, whereas deletion to −108 results in CAT activity similar to that observed with the deletion to −161. These results demonstrate that the dopamine β-hydroxylase gene contains one or more strong positive regulatory elements between residues −230 and −161, and a negative regulatory element between −161 and −129.

**The Dopamine β-Hydroxylase Basal Regulatory Element Stimulates Transcription from a Heterologous Promoter**—In order to further assess the role of the segments of the dopamine β-hydroxylase gene found to be important for basal activity, an oligonucleotide was synthesized corresponding to bases −151 through −180 and cloned in the polylinker region of the pFEl vector, which contains the CAT transcription unit under control of the thymidine kinase promoter of the herpes simplex virus (Edlund et al., 1985). The region between −151 and −180 was chosen for further evaluation because of the results described in Fig. 4 and also because of the strong similarity between the sequence TGCGTCA of the dopamine β-hydroxylase gene and the ENK-CRE2 region of the human proenkephalin gene. The proenkephalin gene is co-expressed with the dopamine β-hydroxylase gene in the adrenal medulla (Winkler et al., 1986), and the ENK-CRE2 region has been shown to play a role in the basal and cyclic-AMP induced expression of the proenkephalin gene (Comb et al., 1986, 1988). Recombinants were constructed which contained multiple copies of this oligonucleotide sequence. These recombinants were then further modified such that the dopamine β-hydroxylase genetic elements were directly adjacent to the thymidine kinase promoter.

The ability of the dopamine β-hydroxylase −180/−151 segment to stimulate transcription from the heterologous promoter was then assessed by transfection of these recombinants into cultured cells. When transfected into SHSY-5Y cells, a recombinant containing two copies of the dopamine β-hydroxylase basal regulatory element, designated DB1(2)-TK-CAT, produced a 5-fold enhancement of CAT activity relative to the TK-CAT vector (Fig. 5). A similar increase in reporter gene activity was observed when DB1(2)-TK-CAT was transfected into PC12 cells, and a marked 20-fold increase in transcription was observed when transfected into the cho-
riocarcinoma JEG-3 cell line. No enhancement was observed when this recombinant was transfected into CV-1 or C6 cells, and, in fact, CAT activity was moderately reduced. These results demonstrate that the segment of the dopamine \(\beta\)-hydroxylase gene corresponding to bases \(-180\) to \(-151\) can function as an enhancer in a cell type-selective pattern. The cell type selectivity extends to three neuroendocrine cell lines, and, in fact, CAT activity was moderately reduced. These results demonstrate that the segment of the dopamine \(\beta\)-hydroxylase gene which plays a role in the cell type-specific expression was evaluated using the electrophoretic mobility shift assay (EMSA). A \textit{Styl/EcoRI} fragment, corresponding to bases \(-87\) to \(-273\), was radiolabeled at the 5'-termini with \([\textit{32P}]\text{ATP}\) and incubated with nuclear extracts prepared from SHSY-5Y, CV-1, or C6 cells. The formation of DNA-protein complexes was resolved by electrophoresis in a nondenaturing polyacrylamide gel. The interaction of the radiolabeled DNA with protein derived from nuclear extracts of SHSY-5Y cells produces a pattern of two distinct bands with the EMSA assay (Fig. 6A), both of which are reduced in intensity when an excess of unlabeled fragment is added as competitor (Fig. 6A, lane 9).

The double-stranded oligonucleotide segment corresponding to DB1 (see Fig. 1A) was used as a competitor in the EMSA in order to identify DNA-protein complexes which correspond to this segment. The presence of an excess of double-stranded DB1 oligonucleotide in the binding reaction with the radiolabeled \textit{Styl/EcoRI} fragment from the dopamine \(\beta\)-hydroxylase gene resulted in a specific reduction in intensity of the lower band, designated as Complex 1 (Fig. 7A). When DNA is incubated with extract from C6 or CV-1 cells a similar pattern is visualized on the EMSA as is observed with extract from SHSY-5Y cells (Fig. 6B). Complex 1 is present when binding reactions are performed in extracts from all three cell lines shown in Fig. 6B and is competed with DB1 oligonucleotide in all cases. These results suggest that there is a nuclear factor common to many cell lines that interacts with this region of the dopamine \(\beta\)-hydroxylase gene.

Several transcriptional regulatory proteins, and the DNA recognition sequence of these proteins, have been previously identified and characterized. In order to evaluate the interaction of some of the more common characterized factors with the dopamine \(\beta\)-hydroxylase 5' flanking sequences, the EMSA assays were performed in the presence of several oligonucleotides that carry the consensus sequence for known transcriptional activators. Due to the similarity between the TGCGTCA segment of the DB1 element and the cyclic AMP (CRE) or phorbol ester response elements of several other genes, oligonucleotides corresponding to the recognition site of transcription factors CREB (see Montminy et al. (1990)) and AP1 (Angel et al., 1987) were added as competitors in the EMSA. No competition of Complex 1 formation is seen with the addition of an excess of oligonucleotide containing the CRE sequence of the somatostatin gene or the AP1 element of the metallothionein gene (Fig. 6, A and C). In addition, the intensity of Complex 1 is not diminished by the addition to the assay mixture of an excess of oligonucleotides containing consensus sequences to transcription factors SP1, AP3, OCT1, or NF-\(\kappa\)B (data not shown). Since the intensity of band 1 is not reduced by the addition of any of the competitor oligonucleotides for known transcriptional modulators, the identity of the protein is unknown at present.

In order to further characterize the interaction of the DB1 segment with nuclear DNA-binding proteins, the oligonucleotide corresponding to the DB1 sequence was radiolabeled and used as a probe for EMSA. The probe forms DNA-protein complexes with nuclear extracts from all cell lines used in the transfection experiments (Fig. 7A). When extracts from JEG-3, SHSY-5Y, or PC12 cell lines are incubated with DB1 oligonucleotide, two bands of shifted mobility are apparent. The migration of the upper band is the same for all three cell lines, whereas the lower band migrates somewhat more slowly in extracts from SHSY-5Y cells than in that from PC12 and JEG-3. Extracts from the two cell lines that do not exhibit enhancer activity produced a different pattern in EMSA, CV-1 cell extract has considerably lower abundance of the complex represented by the upper band but contains two additional complexes, migrating to lower positions. The C6 extract contains only a single complex, migrating to the upper position. All of the DNA-protein complexes observed are reduced with the addition of an excess of unlabeled competitor DNA.
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A DB1 Competitor: Exon: 3 J!G-,3 I PC12 PHSY-SYI C6 -+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+-+

FIG. 7. Interaction of the DB1 fragment, corresponding to $-180/-151$, with nuclear factors. A, 5000 cpm of radiolabeled, double-stranded DB1 oligonucleotide was incubated with 3 $\mu$g of nuclear extract from JEG-3 (lanes 2 and 3), PC12 (lanes 4 and 5), SHSY-5Y (lanes 6 and 7), CV-1 (lanes 8 and 9) or C6 (lanes 10 and 11) cells. In addition, samples in lanes 3, 4, 7, 9, and 11 contained 200 ng of unlabeled DB1 oligonucleotide. B, 5000 cpm of radiolabeled DB1 oligonucleotide was incubated with 3 $\mu$g of nuclear extract from SHSY-5Y cultures. In addition, samples contained 200 ng of competitor oligonucleotide corresponding to DB1 (lane 3), CRE (lane 4), or AP1 (lane 5).

bearing the same sequence. As was observed with the larger, $-87/-273$ probe (Fig. 6C), no competition of DB1-specific complex formation is observed with the addition of oligonucleotide containing CRE or AP1 sites (Fig. 7B). These experiments demonstrate that the DB1 oligonucleotide sequence, which is important in maintaining the expression of the 5' DBH-CAT constructs in the cultured cells, forms sequence specific complexes with nuclear factors from several cell lines. The pattern of complex formation differs between those cell lines in which DB1 acts as an enhancer and those in which the DB1 sequence is inactive.

The DBH Gene Is Regulated by Second Messenger Pathways—In animals, an external stimulus such as immobilization stress will elicit an increase in dopamine $\beta$-hydroxylase activity and mRNA (DBH mRNA) levels in the adrenal (Kvetnansky et al., 1971; McMahon et al., 1992). To begin understanding the intracellular events which mediate changes in DBH mRNA levels in vivo, we have examined the influence of second messenger pathways on DBH gene expression in cultured cells. SHSY-5Y cells were treated with either an analog of cyclic AMP, CPS-CAMP, which activates the cyclic AMP-dependent protein kinase, or with phorbol myristate acetate (PMA), which stimulates the protein kinase C pathway. After 6 h of treatment with effectors, the level of DBH mRNA in total cellular RNA was analyzed. In the experiment presented in Fig. 8A, treatment of cultures with CPS-cAMP resulted in a 1.6-fold increase in DBH mRNA level, while a 2.5-fold increase was observed in RNA extracted from cells treated with PMA. The simultaneous treatment of cells with the two inducers produced a 7-fold increase in the level of DBH mRNA, which is greater than the sum of induction observed with either agent alone.

FIG. 8. The DBH gene is responsive to cyclic AMP and phorbol ester. A, cultures of SHSY-5Y cells were incubated overnight in culture media supplemented with 2% fetal calf serum and subsequently treated with 0.2 mM CPS-cAMP or 0.1 $\mu$M PMA for 6 h. 10 $\mu$g of total RNA was analyzed by Northern blot as described under "Experimental Procedures." B, SHSY-5Y cultures were transfected with 5 $\mu$g of plasmid DNA representing various 5'-DBH-CAT constructs. The following day media was changed so that serum concentration was reduced to 1%, and inducers were added at the concentrations described above. 20 h later, cultures were harvested and assayed for CAT activity. Values presented were standardized by protein and represent the mean $\pm$ S.E. of activity from three individually transfected cultures. The values are presented as the fold increase of treated over untreated cultures, with the mean value for untreated cultures standardized to 1.0 for each plasmid. This experiment has been performed three times with quantitatively similar results. C, SHSY-5Y cultures were transfected with 5 $\mu$g of TK-CAT or DB1(2)-TK-CAT plasmids. Cultures were subsequently treated and data was analyzed as described in A and B.

To investigate whether the change in DBH mRNA levels in response to these agents is mediated through regulatory elements present in the segment of the DBH gene comprising $-395$ to +14, SHSY-5Y cultures were transfected with 5'-DBH-CAT ($-395/+14$) and then treated with either CPS-cAMP or PMA. CAT activity was increased 1.8-2.0-fold when
either agent was present alone, and a 3-fold increase was observed when the inducers were present simultaneously (Fig. 8B).

The second messenger responsive sites were mapped by evaluation of the ability of 5'-DBH-CAT constructs containing progressive deletion of 5'-flanking sequence to mediate induction by cyclic AMP or phorbol ester. Deletion of sequence between -280 and -232 results in a marked increase in the magnitude of induction in cultures incubated with PMA alone or with PMA plus CPS-cAMP. In the experiment presented in Fig. 8B, CPS-cAMP treatment of cultures transfected with either 5'-DBH CAT(-232/+14) or 5'-DBH CAT(-189/+14) resulted in a 3-fold increase in CAT activity, while treatment with PMA elicited a 6-7-fold increase. The simultaneous addition of the two agents resulted in a 12-17-fold increase in CAT activity. These results demonstrate that the ability of the inducers to activate gene transcription is augmented when the 5'-flanking sequence between -295 and -232 is deleted. The presence of the two effectors in combination elicits a synergistic response.

Further deletion of 5'-flanking sequence between -189 and -163 resulted in a reduced ability of effectors to mediate induction of CAT activity. This region is the same segment of DBH which is important for the maintenance of basal activity in SHSY-5Y cells (Fig. 4), and it overlaps the DB1 element.

The DB1 Regulatory Element Is Responsive to Cyclic AMP and Phorbol Ester—The observation that the cyclic AMP and phorbol ester responsive region of the DBH gene is lost upon deletion of DNA sequences overlapping those of the DB1 element (Fig. 8B) prompted us to test the ability of the DB1 element to confer inducibility to these agents. SHSY-5Y cells transfected with the DB1(2)-TK-CAT construct were treated with CPS-cAMP or PMA, and the influence of these agents was determined by measure of CAT activity. These results demonstrate that the ability of the inducers to activate gene transcription is augmented when the 5'-flanking sequence between -295 and -232 is deleted. The presence of the two effectors in combination elicits a synergistic response.

The results of the experiment presented in Fig. 8C demonstrate that modest induction of CAT activity is observed when either inducer is added alone to cultures transfected with DB1(2)-TK-CAT, as compared with the small induction of CAT activity seen with the parental TK-CAT vector. However, when inducers are added simultaneously, CAT activity was increased 12-fold in cultures transfected with DB1(2)-TK-CAT, an induction substantially greater than that observed with the parental TK-CAT vector. These experiments indicate that the DB1 element carries genetic information that will mediate a change in gene expression in response to the combined actions of cyclic AMP and phorbol ester.

**DISCUSSION**

The objective of this study was to identify genetic regulatory elements in the 5'-proximal region of the rat dopamine β-hydroxylase gene which contribute to the specificity of expression of this gene. We have found that a region of the gene containing 395 bases of 5'-flanking sequence confers a cell type-specific pattern of expression onto a reporter gene. The major finding of this study is the identification of a 30-base segment, designated DB1, which is able to function as an enhancer specifically in the neuroendocrine cell lines SHSY-5Y, PC12, and JEG-3. Further evidence that this segment plays a role in the regulation of dopamine β-hydroxylase gene expression is our observation that the region of the DBH gene including the DB1 segment is responsive to stimulation of cyclic AMP and phorbol ester second messenger pathways. The DB1 element plays a dual role in the regulation of DBH gene expression, in that both constitutive and second-messenger induced synthesis are influenced by the presence of this element. The DB1 element exhibits the most potent enhancer activity in the JEG-3 cell line, which is not of catecholaminergic origin, suggesting that genetic regulatory elements similar to DB1 play a role in the expression of other neuroendocrine genes.

Our experiments indicate that the DB1 oligonucleotide could act as a transactivator specifically for neuroendocrine cells; however, in vitro experiments have not detected a DB1 DNA-protein complex that is unique to the cells in which DB1 functions as a constitutive enhancer. Using the 185-base EcoRI-Sty1 fragment from the dopamine β-hydroxylase gene as probe, a similar DNA-protein complex, which is competitive with the DB1 oligonucleotide, is observed in C6 and CV-1 cells, which do not express the dopamine β-hydroxylase gene and do not transactivate the DB1-TK-CAT construct, as well as in the dopaminergic positive SHSY-5Y cell line. Using the DB1 oligonucleotide as probe, several DNA-protein complexes are observed in nuclear extracts from the different cell lines. The presence of several complexes suggests that a variety of proteins interacts with this sequence, and this diversity was not revealed using the 185-base pair probe. These interactions may reflect the presence of multiple binding sites on the DB1 element, or they may reflect numerous protein-protein complexes which bind to a single site. It is noteworthy that the extracts from the three cell lines which have the ability to transactivate DB1, SHSY-5Y, PC12, and JEG-3 exhibit two prominent complexes of similar mobility, whereas extracts from cell lines that do not transactivate DB1 do not possess both of these complexes. This result suggests that two or more DNA-protein complexes may be necessary in order for the DB1 element to function as an enhancer in a cell line.

The DB1 element exhibits functional and sequence similarity to genetic regulatory elements of other neuroendocrine genes. Within the 30-base segment that comprises DB1 is the sequence TGCGTCA. This sequence is similar or identical to several other well-defined regulatory elements, including: 1) the cyclic AMP response element (CRE), which binds transcription factors in the ATF/CREB families and contains the consensus sequence TGACGTCA (see Montminy et al. (1990)) ; 2) the API1 response element, which binds the fos and jun family of transcription factors and contains the consensus sequence TGA(C/G)TCA (see Sheng and Greenberg (1990)); 3) the ENK-CRE2 response element, containing the sequence TGGCTCA, which mediates the response to cyclic AMP of the proenkephalin gene and also plays a role in determining the basal expression of the proenkephalin promoter in CV-1 cells (Comb et al., 1986, 1988); and 4) the FAP element of human c-fos, which can function both as a constitutive and cyclic AMP-mediated transcriptional activator and contains the sequence TGCGTCA (Velich and Ziff, 1990). Whether the activities of the ENK-CRE2 or FAP elements are mediated through interaction with members of the CREB/ATF or fos/jun families has not yet been established, but experiments have been reported which suggest that such interactions may occur. The CRE element of the VIP gene, which will bind to both CREB and fos/jun in vitro (Fink et al., 1991), is competitive with the ENK-CRE2 sequence both in vivo and in vitro (Hyman et al., 1988). In addition, co-transfection of a CREB expression vector with a proenkephalin promoter-reporter construct which included ENK-CRE2 resulted in a
dramatic transactivation of the reporter gene (Huggenvik et al., 1991). Other experiments have indicated a role for junD, a member of the AP1 family, in the induction of the cyclic AMP response of ENK-CRE2 (Kobierski et al., 1991). These studies demonstrate the presence of a bifunctional genetic regulatory element in several neuroendocrine genes, which is important for both basal and second messenger-induced gene expression, and contains a consensus sequence similar to DB1.

Because of the similarity in the sequence of the various CRE/AP1 elements and the DB1 element of the rat dopamine β-hydroxylase gene, we performed several experiments to test whether the CRE or AP1 elements could compete for factors which interact with DB1. The interaction of the DB1 region of the rat dopamine β-hydroxylase gene with nuclear factors is not diminished when excess competitor oligonucleotides corresponding to the CRE element (TGACGTCGA) or the AP1 element (TGAGTCA) are added to the EMSA. These results suggest that the factors which are interacting with the dopamine β-hydroxylase gene in the unstimulated cells are distinct from the well-characterized CREB/ATF or AP1 families. In addition, oligonucleotides corresponding to the recognition sequences for several other transcriptional modulators did not interfere with the binding of the DB1 complex to the nuclear factors, suggesting that the factor(s) interacting with DB1 do not correspond to any of these previously identified proteins and may represent an uncharacterized transcription factor.

One characteristic of the response of the DBH gene and the DB1 element to CPS-cAMP and PMA is that the extent of induction of reporter gene activity or DBH mRNA is synergistic when both inducers are added simultaneously to cell cultures. A synergistic response to these second messengers has also been observed in the VIP (Fink et al., 1991), proenkephalin (Comb et al., 1986, 1988), and α-chlorionic gonadotropin (Andersen et al., 1988) genes. In the VIP and proenkephalin genes, as with DBH, a single genetic regulatory element mediates the response to both inducers. Several potential mechanisms could be operative in generating this response. Each second messenger pathway may activate separate transcription factors, which function as more potent inducers of transcription when activated together in cells than separately. This facilitation of the transcription factors could occur through direct protein-protein interactions, such as heterodimer formation, or through binding to adjacent sites on the DB1 regulatory element. Alternatively, the superadditive response may be generated by activation of the same transcription factor through post-translational modification at different sites by each second messenger pathway. Since both of these second messenger pathways act through protein kinases, it is likely that activation could be achieved through protein phosphorylation.

Regulatory factors can function as either positive and negative modulators of transcriptional activity. The evidence presented in this study suggest that DB1 acts as a positive transcriptional activator. Experimental results supporting this conclusion are: 1) the ability of the oligonucleotide to function as a transcriptional enhancer for a heterologous promoter in SHSY-5Y, PC12 and JEG-3, but not CV-1 or C6 cells, and 2) the observation that deletion of this segment from the dopamine β-hydroxylase 5'-flanking region results in a loss of transcriptional activity. The deletion studies suggest that other elements in the dopamine β-hydroxylase gene may function as negative regulators of transcription. The response of the 5'-DBH-CAT construct to CPS-cAMP and PMA is greatly enhanced when the 5'-flanking sequence distal to −230 is deleted. In addition, reporter gene activity is increased when the region between −163 and −131 is deleted.

These observations suggest the presence of binding sites for negative transcriptional modulators in these regions. The 5'-flanking sequence of the rat dopamine β-hydroxylase gene was compared extensively with that of tyrosine hydroxylase (Lewis et al., 1987), the enzyme which converts dopamine to DOPA in the catecholamine biosynthetic pathway. Both of these genes exhibit a cell type-restricted expression, and the tyrosine hydroxylase gene is transcribed in all tissues which express dopamine β-hydroxylase. A comparison of the 5'-flanking sequences from the two genes reveals several regions of sequence similarity (data not shown), but the functional significance of these segments is currently unknown. The rat tyrosine hydroxylase gene contains two regions of similarity to the DB1 regulatory element of the dopamine β-hydroxylase gene: the CRE element TGACGTCGA at −46 and the sequence TCCATGC at −131, which is identical to the 5'-portion of DB1. Deletion of the TCCATGC region of the tyrosine hydroxylase gene did not influence expression from the tyrosine hydroxylase promoter in pheochromocytoma cells, and the element is not conserved between human and rat tyrosine hydroxylase genes (Cambi et al., 1989). No sequence in the dopamine β-hydroxylase 5'-flanking region corresponds to the AP1/E-box element of rat tyrosine hydroxylase (−2121/−187), which influences basal expression of the gene in PC12 cells (Cambi et al., 1989). At this time the importance of the sequence similarities between the two genes is not clear.

In conclusion, the experimental results reported in this study have demonstrated that the 5'-flanking region of the dopamine β-hydroxylase gene contains genetic regulatory information, which restricts expression to a catecholaminergic cell type and mediates responsiveness to the cyclic AMP and phorbol ester second messenger pathways. A genetic dissection of the 5'-flanking region has revealed the presence of a 30-base bifunctional regulatory element. This DB1 element is a constitutive enhancer in neuroendocrine cells and can also function as a cyclic AMP and phorbol ester responsive element in neuroblastoma cells. The results of these experiments suggest that the DB1 regulatory element segment may play an important role in modulating the expression of the dopamine β-hydroxylase gene in vivo. Further studies will focus on the identification of the nuclear factors which interact with the DB1 regulatory element, which will elucidate the role this DNA-protein complex plays in the transcriptional modulation of dopamine β-hydroxylase and other neuroendocrine genes.

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