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The Human Dopamine D5 Receptor Gene: Cloning and Characterization of the 5'-Flanking and Promoter Region^{†,‡}

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Received December 7, 1994; Revised Manuscript Received February 15, 1995[§]

ABSTRACT: Genomic and overlapping cDNA clones encompassing the entire 5'-untranslated region of the human D5 receptor gene were cloned and sequenced. Comparison of these human D5 receptor genomic and cDNA clones revealed the presence of two exons separated by a small and variably sized intron (of either 179 or 155 bp). We have determined that the major site of transcription initiation of the D5 gene is 2125 bp upstream from the translational initiation start site. The region 5' to the transcription initiation site lacked conventional TATA and CAAT sequences, but contained several putative binding sites for transcription factors, such as Sp1 and Ap1. Luciferase reporter gene constructs containing D5 gene sequence information up to 500 bp 5' of the transcription initiation site were able to stimulate transcription only in SK-N-SH cells but not in COS-7, CHO, P19EC, NB41A3, and SK-N-MC cell lines. Promoter deletion analysis indicated that the D5 gene promoter contained a positive modulator at 119–182 and a negative modulator 251–500 bases upstream from the site of transcription initiation. In addition, in order to detect the expression of functional D5 receptor mRNAs and not those of its expressed pseudogenes, *in situ* hybridization analysis of monkey and human brain using a 5' D5-specific riboprobe revealed that D5 receptor mRNA was most abundant in discrete cortical areas (layers II, IV, and VI), the dentate gyrus, and hippocampal subfields with very little message detected in the striatum. Unexpectedly, D5 mRNA antisense riboprobes labeled discrete cell bodies in the pars compacta of the substantia nigra. The characterization of the genomic organization of the D5 receptor gene and of those factors involved in its transcriptional regulation may aid in our understanding of the role this gene product plays in the generation and maintenance of dopamine D1-like receptor-mediated events.

To date, five distinct genes have been isolated which encode members of the mammalian dopamine (D)¹ receptor family. These receptors have been designated D1/D1A, D2_L,

D2_S, D3, D4, and D5/D1B (Dearry et al., 1990; Zhou et al., 1990; Sunahara et al., 1990, 1991; Bunzow et al., 1988; Giros et al., 1989; Monsma et al., 1989; Sokoloff et al., 1990; Van Tol et al., 1991). D5 belongs to the D1-like class of dopamine receptors (D1/D1A, D1B) that stimulate adenylyl cyclase activity. Both the D1 class of receptors and the D2 class (D2, D3, D4) that inhibit adenylyl cyclase are primary targets of drugs used to treat psychomotor disorders such as schizophrenia and Parkinson's disease (Lee et al., 1978a,b). With the use of radioligand binding techniques, it appeared as though D1 was restricted to primarily motor and limbic regions of the brain (Niznik, 1987). Furthermore, recent studies have suggested that the D1 receptor mediates the psychomotor effects of some drugs of abuse such as cocaine (Xu et al., 1994). The dopamine D5 receptor displayed a pharmacological profile similar to that of the D1 receptor except that it exhibited a 10-fold higher affinity for dopamine. The lack of D5-specific ligands makes it impossible, at present, to distinguish both the pharmacological and functional correlates of D5 receptor stimulation from those of the D1 receptor *in vivo*. Furthermore, the human genome contains two D5 receptor pseudogenes (Grandy et al., 1991; Weinshank et al., 1991; Nguyen et al., 1991a) that have been shown to be transcribed (Grandy et al., 1991; Nguyen et al., 1991b). The existence of transcriptionally competent pseudo-

* This work was supported in part by grants from the MRC of Canada (PG-11121), the Ontario Mental Health Foundation and the Ontario Friends of Schizophrenics. T.V.B. was supported by an Ontario Ministry of Health Personnel Development Research Fellowship. J.H.M.-W. is the recipient of an NIMH Research Scientist Development Award (MH00818), and was also supported by a grant from The Stanley Foundation. H.B.N. is a recipient of a NARSAD Established Investigator Award and is a Career Scientist of the Ontario Ministry of Health.

[†] The nucleotide sequence(s) reported in this paper has (have) been submitted to the GenBank/EMBL Data Bank under Accession Number(s) U21164.

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[§] Abstract published in *Advance ACS Abstracts*, April 15, 1995.

¹ Abbreviations: RT, reverse transcriptase; PCR, polymerase chain reaction; UTR, untranslated region; 5'-RACE, 5'-rapid amplification of cDNA ends; D, dopamine; AP-1, activator protein 1; GHF-1, growth hormone factor 1; GRE, glucocorticoid responsive element; ERE, estrogen responsive element; A, amygdala; C, caudate; P, putamen; CL, claustrum; DG, dentate gyrus; S, subiculum; erc, entorhinal cortex; neo, neocortex.

genes could potentially frustrate attempts to determine both the quantification and tissue-specific distribution of D5 dopamine receptor mRNAs in humans.

The 5'-flanking regions of the dopamine D1/D1A and D2 genes have been cloned and their transcription initiation sites determined (Minowa, M. T., et al., 1992; Minowa, T., et al., 1992; Zhou et al., 1992; Valdenaire et al., 1994). During the course of these efforts, it was discovered that the D1 receptor gene, formerly considered to be intronless, possessed a small intron in its 5'-untranslated region, with a transcriptional start site approximately 1 kilobase (kb) from the start of its coding sequence. In contrast, the D2 gene possessed an extremely large 5' intron, the length of which remains to be determined, but has been reported to be in excess of 50 kb (Bunzow et al., 1988). The promoters for these genes lack conventional CAAT and TATA sequences, but each possesses "GC" boxes reminiscent of "housekeeping" gene promoters [for review, see Mouradian et al. (1994)]. However, the D1 and D2 genes are both tissue-specific and under a high degree of transcriptional regulatory control (Mouradian et al., 1994; Minowa et al., 1993; Minowa et al., 1994).

In order to assess the relative importance of the D5 receptor it was necessary to determine the molecular mechanisms that govern its expression and its neuroanatomical distribution. In this report, we describe the molecular cloning of the 5'-flanking region of the D5 receptor gene, the identification of its transcriptional start site and promoter structure, and the development of a 5'-untranslated D5-specific riboprobe for mapping the neuroanatomical distribution of human messenger RNAs via *in situ* hybridization.

MATERIALS AND METHODS

Genomic Cloning. To obtain clones of the 5'-flanking region of the human D5 receptor gene, a lambda-EMBL 3 SP6-T7 human genomic library (Clontech, Palo Alto, CA) was screened with a 2.1 kilobase (kb) (*AccI-SacI*) fragment encoding the amino terminus and a portion of the 5'-untranslated region (UTR) of the D5 gene under high stringency conditions (Sunahara et al., 1991). Restriction map and Southern blot analysis of positive clones revealed a 3.6 kb (*XbaI*) fragment corresponding to the 5'-UTR of the human dopamine D5 receptor gene. This 3.6 kb (*XbaI*) fragment was subcloned into pSP73 (Promega, Madison, WI), and sequenced in both directions by the dideoxy chain-termination method described by Sanger et al. (1977) using the Sequenase version 2.0, 7-deaza-dGTP sequencing kit (United States Biochemical, Cleveland, OH).

cDNA Cloning. cDNA clones were obtained by a combination of 5'-Rapid Amplification of cDNA Ends (5'-RACE) and expression of the D5 gene in COS-7 cells followed by reverse transcription and PCR. Human brain samples were obtained from the Canadian Brain Tissue Bank, and all post-mortem times (death to freezing interval) were less than 24 h. Total RNA was isolated from human post-mortem cortical brain tissues by the method of Chomczynski and Sacchi (1987). 5'-RACE was performed using a 5'-RACE System (Gibco/BRL, Gaithersburg, MD) under conditions essentially as described by the manufacturer. In brief, less than 1 μ g of total RNA was DNased for 1 h at room temperature and heat-inactivated at 90 °C for 5 min. The RNA was then reverse-transcribed with Superscript RT at 37 °C using the

D5-specific primer P1 (5'-GTAGCGGTCCACGCTGAT-GACGCA-3'), encoding complementary bases +416 to +394 of the D5 coding region. Single-stranded cDNAs were purified with a Glass-Max spin cartridge and tailed by terminal deoxynucleotidyltransferase with poly(dC). To identify the point of transcription initiation, cDNAs were subjected to two rounds of PCR amplification, using primer P2 (5'-GAAAGGGATGTGACAGCAGCCAGCTGCAT-3'), complementary to bases -1261 to -1290, and the manufacturer-provided anchor primer and then reamplified with P3 (5'-CCTCCTCCATCCTGTTGATGGTGGCACCT-3'), complementary to bases -1747 to -1776, and the anchor primer (see Figure 2A). PCR conditions were 95 °C for 5 min and 85 °C for 2 min, at which time 2.5 units of *AmpliTaq* were added to each reaction. Samples were denatured at 95 °C for 30 s; primer (30 pmol) was annealed at 55 °C for 30 s, and extended at 72 °C for 1 min for 30 cycles, followed by 10 min at 72 °C. All clones obtained were subcloned into pSP-73 and sequenced in both orientations. Other overlapping cDNAs were obtained in a similar fashion using the nested D5-specific oligonucleotides P4 (5'-CATGAA-CATAGTCACATTCAAGG-3'), complementary to bases -1853 to -1833, and P5 (5'-AAGCCGTCTAGGATGAG-GTGT-3'), complementary to bases -876 to -896 of the 5'-flanking region of the D5 gene (see Figure 2A).

Expression in COS-7 Cells. To obtain cDNA clones in the "GC"-rich region of the 5'-UTR flanking bases -896 and the coding region of the D5 gene, a 5.7 kb (*EcoRI*) genomic fragment, encompassing the entire transcriptional unit of the D5 gene, was subcloned into the expression vector pCD. This construct was transiently transfected into COS-7 cells using a polyamine-mediated transfection (Lipofectamine, Gibco/BRL) method (see below). Total RNA was isolated, as described above, and reverse-transcribed with 50 units of M-MLV reverse-transcriptase (GeneAMP RNA PCR kit; Perkin Elmer/Cetus) and 7-deaza-dGTP/dGTP (3:1), as per manufacturer's instructions. Single-stranded DNA was subjected to one round of PCR, again with 7-deaza-dGTP/dGTP, using oligonucleotide primers P6 (5'-GGTG-GTGGGGAAAAGGACAATCTC-3') complementary to bases -1009 to -986, and P7 (5'-CACGCCAGAGACACGAT-GAAGAC-3'), complementary to bases +154 to +131, or oligonucleotides P8 (5'-AAGCGTGGCCAGGGCTAAGG-3'), complementary to bases -641 to -623, and P9 (5'-CTCGCTGACAGCCAGGGCTTCT-3'), complementary to bases -247 to -270 (see Figure 2A). All clones obtained were subcloned into pSP-73 and sequenced in both orientations as described above.

D5-Luciferase Plasmid Constructs and Assay. A segment of the D5 5'-flanking region, encompassing 500 bases upstream of the transcription initiation site and 197 bases downstream of this position, was generated by PCR of a 1.2 kb (*XbaI*) fragment, using oligonucleotide primers directed to the T7 transcription initiation site of pSP-73 and an internal primer 197 bp downstream of the D5 transcription initiation site (P10, 5'-ATGCAAGGTCTTCCCTCATATTG-3'). This fragment was then blunt-ended, followed by restriction digestion at the 5'-end with *XbaI*, and subcloned into the luciferase gene-containing plasmid PL(KS)b-LUCnPL to create p500D5-LUC. The deletion mutants, p182D5-LUC and p51D5-LUC, were created by digesting 500D5-LUC with *HindIII* or *EcoRI*, respectively, followed by religation. p251D5-LUC was generated by PCR using oligonucleotide

primers P11 (5'-ATCCACCCACCTCGGCCTCCAAA-3') and P25, and p119D5-LUC was created in a similar fashion using oligonucleotides P12 (5'-GGTAAAACACATATGG-GA-3') and P10 (see Figure 1).

Several mammalian cell lines were tested for luciferase activity. The murine neuroblastoma cell line NB41A3 was maintained in Ham's F-10 medium, supplemented with 15% horse serum and 2.5% fetal bovine serum. Murine P19 EC, rat COS-7, human neuroblastomas SK-N-MC and SK-N-SH, and Chinese hamster ovary (CHO) cells were maintained in α -minimal essential medium supplemented with 10% fetal bovine serum. Cells were grown at 37 °C in 10% CO₂ in a humidified incubator. Transfections were carried out using Lipofectamine (Gibco/BRL), using the manufacturer's protocol. In brief, for a 100 mm plate, 10 μ g of the heterologous D5-luciferase constructs or luciferase plasmid alone, together with 3 μ g of the expression vector CMV- β GAL, was incubated with 100 μ g of Lipofectamine at room temperature for 45 min in 1 mL of sera-free media. Cells were washed with serum-free media and the transfection mixture was added to each plate in a total volume of 5 mL. After 5 h, cells were washed with phosphate-buffered saline and maintained with the appropriate media supplemented with sera. Twenty-four hours after transfection, cells were harvested in 300 μ L of lysis buffer, containing 50 mM TRIS/2-(*N*-morpholino)ethanesulfonic acid (MES), pH 7.8, 1 mM DTT, and 0.1% Triton X-100. Cell lysates were vortexed for 10 s and briefly spun at 14000*g* in an Eppendorf 5415 C microcentrifuge to pellet cellular debris. Using a Bio-Orbit 1250 Luminometer, 200 μ L of cell lysate together with 15 μ L of 750 mM TRIS/MES (pH 7.8), 150 mM MgOAc, and 40 mM ATP was mixed with 200 μ L of 1 mM luciferin in 5 mM potassium phosphate, and luciferase activity was measured. All values obtained were normalized to the β -galactosidase activity of the extract. β -Galactosidase activity determinations were performed essentially as described by Sambrook et al. (1989).

Cell lines were subjected to RT/PCR in an attempt to identify those that express the D5 gene. RT/PCR was performed as described above using oligonucleotides D1b-1 (5'-TCGTCACTCATCAGCTTCTACATC-3') and D1b-2 (5'-ATTGAGAGAGGAGTTGGCCCAGCC-3') directed to homologous coding regions of both the human D5 and rat D1b receptor genes. Purified single-stranded cDNAs were subjected to the PCR using primers under conditions described under cDNA Cloning.

In Situ Hybridization of D5 Receptor mRNAs. The brains of Old World monkeys (*Macaca nemestrina*) were obtained from the Regional Primate Research Center at the University of Washington. These animals had been sacrificed as part of other protocols that did not require study of the brain. Human brain samples were obtained at autopsy, and tissue blocks were excised and rapidly frozen on dry ice. Post-mortem intervals of samples were less than 24 h. Tissue samples were maintained at -80 °C until processed for *in situ* hybridization.

Sections were removed from frozen storage and immersed in 4% formaldehyde for 60 min. *In situ* hybridization was performed as previously described (Meador-Woodruff et al., 1991, 1993). Briefly, sections were hybridized with ³⁵S-labeled riboprobes [(10–20) \times 10⁶dpm/section] in 75% formamide hybridization buffer, and incubated overnight at 55 °C. Riboprobes were synthesized from a plasmid

consisting of a 198 bp insert, encoding the 5'-termini of the D5 receptor gene cDNA, cloned into pGEM-7Zf(+) using T7 RNA polymerase. Following hybridization, sections were treated with RNase A and then rinses of increasing stringency, culminating in a 60 min wash in 0.5× SSC at 55 °C. The slides were then dehydrated and exposed to Kodak X-OMAT film for 4–6 weeks. Control slides, including both the "sense"-strand and RNase-pretreated "antisense"-labeled sections run in parallel with "antisense"-labeled sections, were processed as previously described (Meador-Woodruff et al., 1991, 1993).

RESULTS AND DISCUSSION

In this report, we describe for the first time the genomic organization of the 5'-flanking region and promoter structure of the human dopamine D5 receptor gene. We also provide the first *in situ* hybridization data of the human D5 receptor gene using 5' probes distinct in nucleotide sequence from the two D5 pseudogenes. As such, we provide the first unequivocal data on the cell-specific distribution of the D5 mRNA in human and Old World monkey brain sections. The characterization of the genomic organization of the D5 receptor gene and of those factors involved in its transcriptional regulation may now aid in our understanding of the role this gene product plays in the generation and maintenance of the dopamine D1-like receptor-mediated events.

Genomic Cloning. In order to identify the transcriptional start site of the dopamine D5 receptor gene, it was first necessary to obtain sequence information for the 5'-flanking region of this gene. A human genomic lambda-EMBL 3 SP6-T7 library was screened with a 2.1 kb (AccI-SacI) fragment encoding a large portion of the intronless coding region and 1278 bp of the 5'-UTR of the D5 gene. This search yielded several positive phage clones, one of which contained a 3.6 kb fragment (*Xba*I) corresponding to the 5'-untranslated region of the dopamine D5 receptor gene which was entirely sequenced. Sequence data for the human genomic D5 receptor clones are depicted in Figure 1. The most 3' 789 bases of the 3.6 kb (*Xba*I) clone were identical to the 5'-portion of the 2.1 kb (AccI-SacI) fragment of the dopamine D5 gene. The UTR sequence 5' to the translational start site of the D5 gene was demonstrated to be very "GC-rich". Also, the 5'-UTR of the D5 gene shared a high degree of homology (95%) with the two related pseudogenes, but lost identity with these pseudogenes 5' of nucleotide -1916 (Marchese et al., 1995). Also of note, there existed 2 antisense *Alu* sequences between nucleotides -3244 to -2953 and -2624 to -2315, 5' to the translational start site (Marchese et al., 1995).

cDNA Cloning. 5'-RACE was performed in order to determine the 5' transcriptional start site of the D5 receptor gene. A restriction map of the 5'-flanking region of the D5 gene illustrates the cDNAs obtained after performing 5'-RACE on total RNA derived from human cortical tissue samples (Figure 2A). After the 5'-RACE reaction was performed (using oligonucleotide P3 as a D5-specific primer), ethidium-stained agarose gel electrophoresis and Southern blot analysis revealed an approximately 380 bp sized band. This cDNA was subcloned into pSP-73 and sequenced. This sequence information indicated that the cDNAs most 5'-terminus corresponded to base -2125 of the genomic gene

FIGURE 1: Nucleotide sequence of the 5'-flanking region of the human dopamine D5 receptor gene. The transcription initiation site obtained by 5'-RACE is denoted by a diamond (◆). Uppercase letters are used for exonic sequences, and lowercase letters in italics are used for intronic sequences. Letters in boldface script italics indicate intron/exon acceptor/donor sites. Lower case letters in light face roman denote promoter and 5'-flanking noncoding sequences. Oligonucleotide primers used and their direction are indicated. Putative cis-acting response elements are shaded, and arrows denote fragments of the D5 5'-flanking region fused to the firefly luciferase gene.

sequence (Figures 1 and 2B). Several other clones were also sequenced, and they all showed the identical 5'-termini. The 5'-RACE assay was repeated once more using total human cortical RNA from a different post-mortem cortical brain tissue sample, again providing an identical result. The region immediately 5' to the transcription initiation site did not contain "CAAT" or "TATA" motifs similar to the D1 and D2 promoters (Minowa, M. T., et al., 1992; Minowa, T., et al., 1992). However, the D5 promoter region is not "GC"-

rich and appears to have one discrete transcriptional start site, in contrast to "housekeeping genes" (Mouradian et al., 1994). This region of the D5 gene also contained multiple transcription factor binding motifs including sites for Sp-1, AP1, estrogen, and glucocorticoid response element half-sites and an 11-base-long sequence identical to the pituitary-specific human growth hormone factor, GHF-1 (Figure 1) (Lefevre et al., 1987). In an attempt to obtain cDNA clones from the entire 5'-UTR, cDNAs from the first-strand

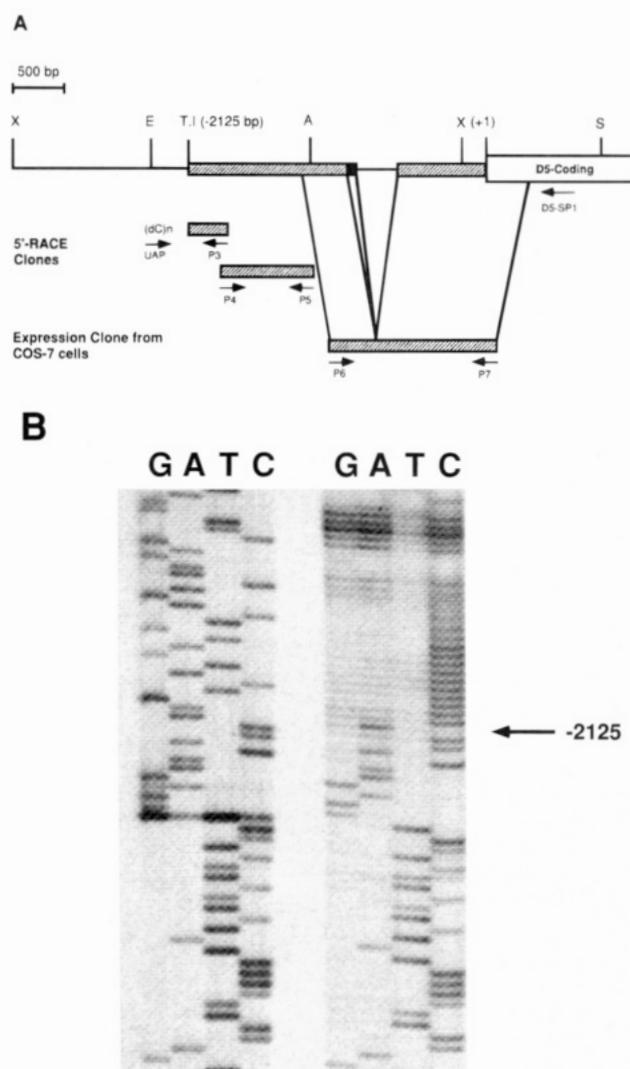


FIGURE 2: *Panel A:* Schematic of the D5 receptor 5'-flanking region. Restriction endonuclease sites are denoted X (*Xba*I), E (*Eco*RI), A (*Apa*I), and S (*Sac*I). The 5' end of the D5 coding region is designated as +1, and the transcription start site (T. I.) determined by 5'-RACE cDNA cloning is at position -2125. Hatched boxes represent cloned cDNAs or exonic sequences, and solid black lines are intronic and 5'-flanking region sequences. Oligonucleotides used to generate the cDNA clones are shown as arrows, and "UAP" represents the universal amplification primer for the 5'-RACE reaction (Gibco/BRL). *Panel B:* Sequence autoradiogram depicting transcription initiation of the D5 receptor. Sequencing of the complimentary strand of the 381 bp RACE-generated cDNA on the right and the sequence of human genomic DNA on the left reveal that the site for transcription initiation corresponds to the thymidine residue at position -2125 bp.

synthesis reaction were subjected to PCR amplification with D5-specific oligonucleotides within this region. Using oligonucleotides P4 and P5 (see Figure 2A), a 978 bp cDNA was obtained that was identical to the D5 genomic sequence from nucleotides -1853 to -876. However, using a 5'-RACE assay system, we were unable to obtain cDNA clones corresponding to the area between nucleotide -876 of the genomic sequence and the coding region.

cDNA Cloning by Expression in COS-7 Cells. Obtaining cDNA clones of the D5 5'-UTR between nucleotide -876 and coding may have been complicated by several factors. Messenger RNA of the D5 gene was reported to be rare, and the high identity between the D5 receptor gene and the two related pseudogenes interfered with conventional cloning

techniques. Homology screenings of different human brain cDNA libraries in order to clone a cDNA from the 5'-flanking region of the D5 gene were unsuccessful. Several attempts were made to obtain cDNA clones encompassing the region between nucleotide -876 and coding using single-stranded cDNAs produced from conventional RT-PCR and 5'-RACE assay techniques. Although several clones were obtained, none were identical and were considered to be artifactual. As stated, the area immediately 5' to the D5 coding region was very "GC"-rich, and all clones obtained appeared to contain "introns" of differing lengths. This was due, we suspect, to the large amount of secondary structure formed in this region of the gene and may have accounted for our lack of success using conventional cloning techniques.

In order to obtain cDNA clones between nucleotide -876 and coding, we circumvented the problems of scarce human brain samples, low mRNA expression, high "GC" content, and the existence of transcriptionally competent pseudogenes by inserting a 5.7 kb (*Eco*RI) genomic fragment containing the entire coding and 5'-UTR of the D5 receptor gene into the expression vector pCD. This clone was transfected and expressed in COS-7 cells; total RNA was isolated and subjected to RT-PCR using 7-deaza-dGTP. Using either oligonucleotides P6 and P7 or oligonucleotides P8 and P9, and 7-deaza-dGTP (Figure 2A) to amplify single-stranded cDNA clones obtained by reverse transcription in this region, one specific band in each case was identified by Southern blot analysis. Sequence analysis of the cDNA clones obtained overlapping the region between nucleotide -876 and the coding region demonstrated for the first time that the D5 gene has at least two exons and that splice variants for the D5 receptor gene existed. Six clones were sequenced, five of which indicated that the intron in the 5'-UTR used "CGGT" (at position -492) as the primary splice donor site with one clone indicating "AGGT" as a splice donor site (splice junction position at -468). All clones obtained used the identical acceptor site at position -313 (Figure 1). These data indicated that D5 has two splice variants, one of these has a 5' intron of 179 bases and another with a 5' intron of 155 bases. As such, exon 1 was either 1633 or 1657 bp in length and exon 2 extended beyond the 3'-termini of coding. A small 5'-UTR intron was also reported in the D1 dopamine receptor gene (116 bp) in the same region (Minowa, M. T., et al., 1992). Both of the donor sites observed in the D5 cDNA clones are proper splice donor sites according to Mount (1982).

Transient Transfection of D5-Luciferase Constructs. The cloned D5 gene promoter sequences were fused into the luciferase gene-containing plasmid PL(KS)b-LUCnPL to demonstrate that this region of the D5 gene could drive transcription in the presence of the necessary nuclear factors. The results of the luciferase assays using this region and also deletion mutants of the 5' flanking sequences of D5 in SK-N-SH cells are presented in Figure 3. Part A is a schematic of the deletion mutants fused in front of the firefly luciferase gene. PL(KS)b-LUCnPL (pKS-LUC on graph), which is a promoterless plasmid, was used as the negative control. The greatest increase (3.5 \times) of luciferase transcription was seen with the plasmid p251D5-LUC, with almost as high a level of activity with plasmid p182D5-LUC. The transcriptional activity of the longest fragment, p500D5-LUC, was about half of that seen with p251D5-LUC. These data may indicate the presence of a negative modulator (silencer)

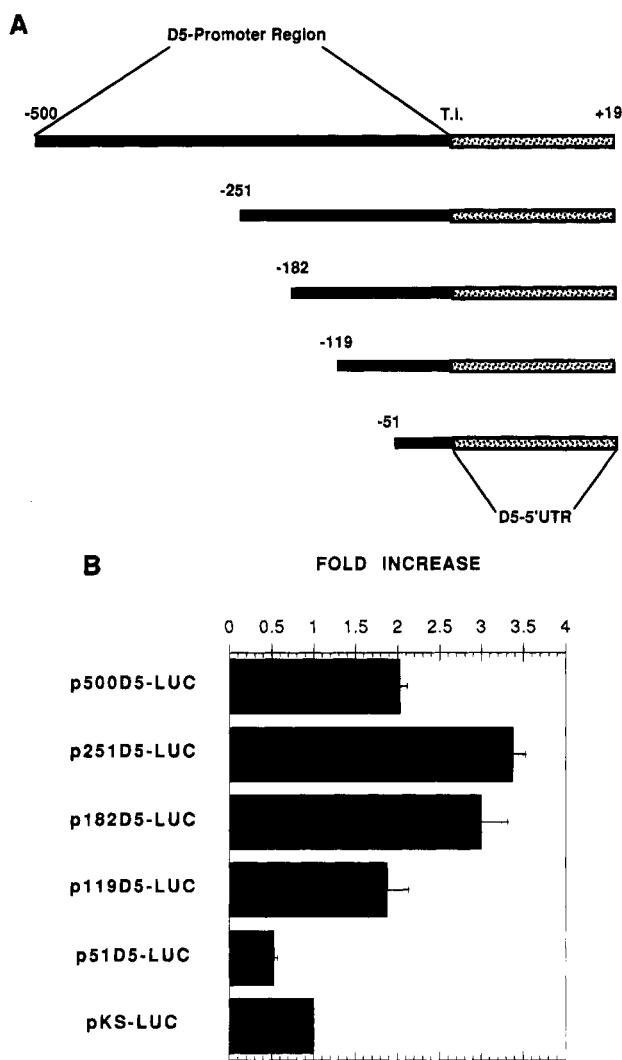


FIGURE 3: *Panel A:* Schematic representation of chimeric D5 promoter deletion mutants/firefly luciferase gene constructs. All deletion mutants fused into the heterologous luciferase vector PL-(KS)b-LUCnPL (pKS-LUC in panel B) contained the first 197 bases of exon 1 (stippled region) and either 500, 251, 182, 119, or 51 bases corresponding to the genomic sequence immediately 5' to the transcriptional start site (solid black bars). The negative control, pKS-LUC, contained no D5 sequences. *Panel B:* Functional analysis of the D5 promoter region. Relative luciferase activity in human neuroblastoma SK-N-SH cells after transient transfection with plasmids pKS-LUC, p500, p251, p182, p119, and p51D5-LUC. These values represent the means of four independent experiments performed in duplicate. The promoterless plasmid pKS-LUC was used as the negative control, and results are represented as fold increase above control values. All values obtained were normalized to the β -galactosidase activity of the extract.

between bases -2626 and -2387, the nucleotide sequence in p500D5-LUC that was deleted in the p251D5-LUC construct (see Figure 3A). By the same token, a positive response element may be present between bases -2307 and -2244 (i.e., the portion of p182D5-LUC that was not present in p119D5-LUC). The activity of the smallest D5 promoter fragment was consistently about half of that seen in the negative control and about 15% of the maximal activity seen with the p251D5-LUC construct. All data are the result of four independent experiments carried out in duplicate using plasmids from at least two different preparations. The D5 gene's promoter region contains several consensus sequences for multiple transcription factors, and we are currently attempting to identify the specific motifs and trans-activating

Table 1: RT/PCR of D5/D1B Receptor mRNA and D5 Promoter-Induced Luciferase Activity in Various Mammalian Cell Lines^a

cell line	species	RT/PCR	luciferase
B 104	rat	—	—
RINM		—	—
rat 1		—	—
C6		—	—
PC 12		—	—
B-TCE	mouse	—	—
NB41A3		—	—
P19EC		—	+
COS-7	monkey	—	—
CHO-K1	hamster	—	—
T671	human	—	—
IMR32		—	—
SK-N-MC		—	—
SK-N-SH		—	+

^a Transfection conditions for each cell line were determined using β -gal activity as the standard for transfection efficiency. All D5 promoter deletion mutants failed to increase basal luciferase activity by a significant amount in all cell lines tested with the exception of the human neuroblastoma line SK-N-SH. RT/PCR was performed using oligonucleotides encoding sequences homologous to both the human D5 and rat D1B receptor genes.

factors responsible for the regulatory changes in activity observed.

D5 promoter activity displayed a cell-specific pattern of expression. The results of our luciferase assays and RT/PCR assays using RNA derived from different mammalian cell lines are presented in Table 1. Six different mammalian cell lines were tested for luciferase activity using the five different D5 promoter deletion mutants to drive the transcription of the luciferase gene. Although optimal transfection conditions were obtained for all cell lines tested, D5 promoter-deletion mutant constructs were unable to increase luciferase transcription significantly above basal levels in SK-N-MC, P19EC, COS-7, NB41A3, and CHO-K1 cells. We failed to detect the presence of D5 messenger RNA in all cell lines tested (see Table 1), and radio-ligand binding assays did not reveal the presence of a D5 receptor population in the SK-N-SH cell line (data not shown). While all our evidence indicates that the SK-N-SH cell line did not express the D5 gene product, this cell line appeared to contain the required factors necessary to interact with specific sequences within the D5 promoter to enhance the transcription of the luciferase gene. Similarly, it has been reported that the D2 promoter can drive the transcription of a heterologous luciferase construct in GH3, C6 glioma, and primary fibroblast cells which do not express the D2 gene (Valdenaire et al., 1994). The D5 gene has been shown to be expressed in human peripheral lymphocytes (Takahashi et al., 1992) and in cells in the lamina propria of the stomach (Mezey & Palkovits, 1992).

In Situ Hybridization. We found the D5 5'-UTR extended 5' of the point of divergence with the two related pseudogenes. We demonstrated that a portion of the D5 cDNA (209 bp) shared no homology with either of the D5 pseudogenes. This region was cloned and used to create a novel D5-specific riboprobe for the detection of D5 mRNA in *in situ* hybridization analysis of both human and monkey brain slices. Table 2 provides a summary of the D5 receptor mRNA distribution in human and Old World monkey brain slices. Figure 4 shows D5 receptor mRNA distribution in Old World monkey brain. Coronal sections of old world

Table 2: Dopamine D5 Receptor mRNA Distribution in Old World Monkey and Human Brain Slices^a

region	monkey	human
basal ganglia		
caudate	++	-
putamen	++	-
claustrum	++	-
globus pallidus	+	-
hippocampus and related structures		
dendate gyrus	+++	+++
hippocampal subfields	++	++
subiculum	-	+
entorhinal cortex	++	+
amygdala	++	-
thalamus (anterior)	+	-
pulvinar	++	-
lateral geniculate	+	-
hypothalamus	+	-
neocortex		
superficial layers	+	+
deep layers	++	++
substantia nigra	+	+

^a Sections of Old World monkey and human brain were incubated with a 5' ³⁵S-labeled 198 bp riboprobe that shared no sequence identity with either of the two human pseudogenes. Areas denoted with "+++" expressed the highest levels of D5 mRNA, areas denoted by "++" contained moderate levels, "+" denotes sparse labeling, and areas denoted with "—" contained no appreciable message.

monkey brain were incubated with a 5' ³⁵S-labeled 198 bp riboprobe that shared no identity with either of its two pseudogenes, under conditions described under Materials and Methods. Panels A, C, and E are darkfield micrographs following hybridization with an antisense probe, and panels B, D, and F are after treatment with a sense-strand probe. Panel A is at the level of the globus pallidus, and shows faint and diffuse labeling in the caudate (C), putamen (P), claustrum (CL), and amygdala (AM), with some faint and patchy labeling in the hypothalamus (HT) and globus pallidus (GP). Diffuse labeling through much of the neocortex can also be appreciated. Panel C is at the level of the thalamus, and shows rather dense labeling in the dentate gyrus (DG) and moderate labeling in the hippocampal subfields (H). Fainter labeling is also present in the tail of the putamen (P), thalamus (Th), and lateral geniculate nucleus (LGN). Also notice the diffuse labeling through most of neocortex, with slight enrichment of labeling in deeper layers. Surprisingly, the pars compacta of the substantia nigra (SN) appears to have faint and specific labeling. Panel E is at the level of the pons, and shows hippocampal labeling as seen in panel C, but also diffuse labeling in the pulvinar (PL), pons (PO), and periaqueductal gray (CG).

Figure 5 depicts D5 receptor mRNA distribution in human brain. Panels A, C, and E are darkfield micrographs following hybridization with an antisense probe, and panels B, D, and F are after treatment with a sense-strand probe. Panel A is at the level of the hippocampus, and is very similar to observations made in the monkey brain. The dentate gyrus (DG) has abundant mRNA, and hippocampal subfields (H) appear to have modest levels of D5 receptor mRNA. Fainter labeling is present in the entorhinal cortex (ERC) and in the subiculum (S). Diffuse labeling is seen throughout the neocortex (N), with some suggestion of enrichment in deeper laminae (small arrows). Panel C is at the level of the midbrain, and shows some labeling in the pars compacta of the substantia nigra (SN), as seen in the monkey. Panel E

is from frontal lobe, demonstrating labeling throughout much of the neocortex. Again, there is a suggestion of slight enrichment in the deeper layers of cortex (small arrows). No specific labeling is seen in the matched sense-strand control sections (panels B, D, and F). Figure 6 provides a more detailed map of D5 receptor mRNA distribution in human brain sections of the hippocampus and neocortex. Part A is at the level of the hippocampus and reveals intense labeling in the granular cell layer of the dentate gyrus (DG). Faint labelling can also be seen in the pyramidal cell layer of CA2, CA3, and CA4 (2, 3 and 4). Somewhat lower levels were observed in CA1 (1), the subiculum (S), the entorhinal cortex (erc), and the neocortex (neo). Part B demonstrates in more detail the labeling seen in neocortex, with the highest D5 receptor mRNAs levels seen in layers II, IV, and VI.

We have been able to develop a D5-specific riboprobe in order to delineate the distribution of the D5 receptor gene expression. D5 receptor mRNA displayed rare and tissue-specific expression patterns in human and Old World monkey brains. Since the original report of the cloning of the D5 receptor gene, little has been done to characterize the distribution of this receptor either by *in situ* hybridization or by immunohistochemical techniques. Furthermore, it was demonstrated that at least one pseudogene was transcriptionally competent (Weinshank et al., 1991; Nguyen et al., 1991b). Because of the high identity between the D5 gene and both of the related pseudogenes, the existence of pseudogene mRNA would give false positive signals in *in situ* hybridization analysis of the D5 message if a riboprobe directed to the coding region was used. We have cloned a region of the D5 gene sequence that shared no identity with either of the pseudogenes (Marchese et al., 1994). By using this region to make riboprobes, the signals seen in human and Old World monkey slices should represent only D5 mRNA.

While our data concerning the distribution of D5 mRNA are consistent for the most part with recently published data (Meador-Woodruff et al., 1994), the lack of identity between 5'-termini of the D5 cDNA and the pseudogenes allows for better resolution of the D5 signal. However, *in situ* hybridization with a D5-specific riboprobe reveals labeling in discrete cells of the pars compacta of the substantia nigra. While unexpected, this result is highly reproducible. Besides the apparent presence of message in the nigra, a similar pattern of distribution was observed for the rat brain for the D1b receptor (Tiberi et al., 1991). However, there are some striking differences. D1b seems to be very abundant in the rat lateral mammillary nuclei and the parafascicular nucleus, signifying a differential mode of expression from the D5 gene. As yet, no report has been published concerning the cloning of the D1b promoter region, and the set of signals governing the transcription of this gene is unknown. In contrast, the pattern of distribution of D5 gene expression in Old World monkey was identical to that observed in human brain slices. Message levels for the human D1 gene were highest in caudate-putamen, nucleus accumbens, and olfactory tubercle (Monsma et al., 1990; Fremeau et al., 1991). However, mRNA for all dopamine receptor gene subtypes has been reported in the granular layer of the dentate gyrus which overlaps with the D5 gene expression (Meador-Woodruff et al., 1994; Fremeau et al., 1991). Aside from its presence in the dentate gyrus, the D5 mRNA distribution seems to be rare, restricted, and distinct from other dopamine

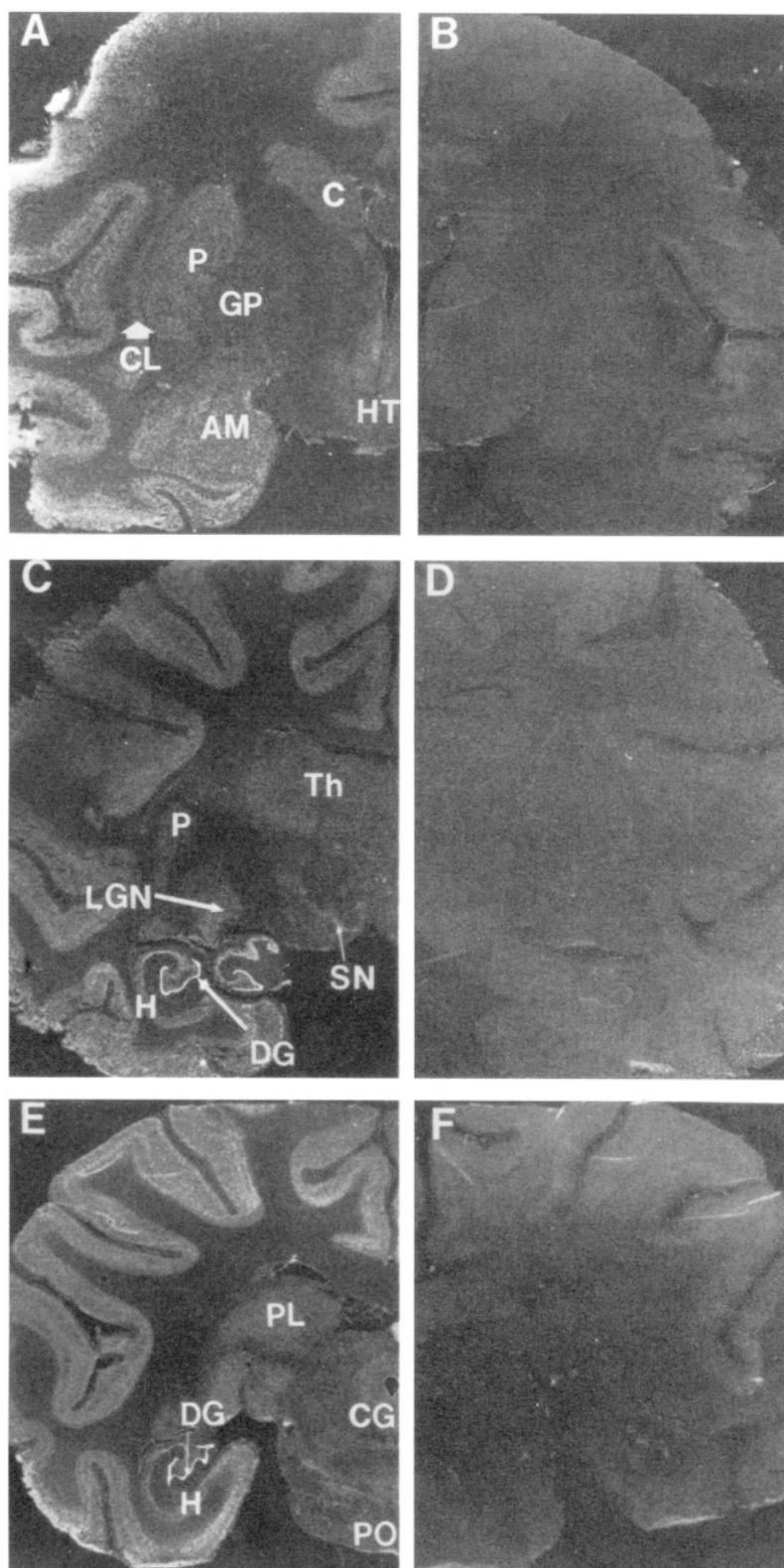


FIGURE 4: *In situ* hybridization of D5 receptor mRNA in Old World monkey brain. Coronal sections of Old World monkey brain were incubated with a 5' ^{35}S -labeled 198 bp riboprobe under conditions described under Materials and Methods. Panels A, C, and E are darkfield micrographs following hybridization with an antisense probe, and panels B, D, and F are after treatment with a sense-strand probe. *Panel A* is at the level of the globus pallidus, and shows faint and diffuse labeling in the caudate (C), putamen (P), claustrum (CL), and amygdala (AM), with some faint and patchy labeling in the hypothalamus (HT) and globus pallidus (GP). Diffuse labeling through much of the neocortex can also be observed. *Panel C* is at the level of the thalamus, and shows rather dense labeling in the dentate gyrus (DG) and moderate labeling in the hippocampal subfields (H). Fainter labeling is also present in the tail of the putamen (P), thalamus (Th), and lateral geniculate nucleus (LGN). Also notice the diffuse labeling through most of the neocortex, with slight enrichment of labeling in deeper layers. Surprisingly, the pars compacta of the substantia nigra (SN) appears to have faint and specific labeling. *Panel E* is at the level of the pons, and shows hippocampal labeling as seen in panel C, but also diffuse labeling in the pulvinar (PL), pons (PO), and periaqueductal gray (CG).

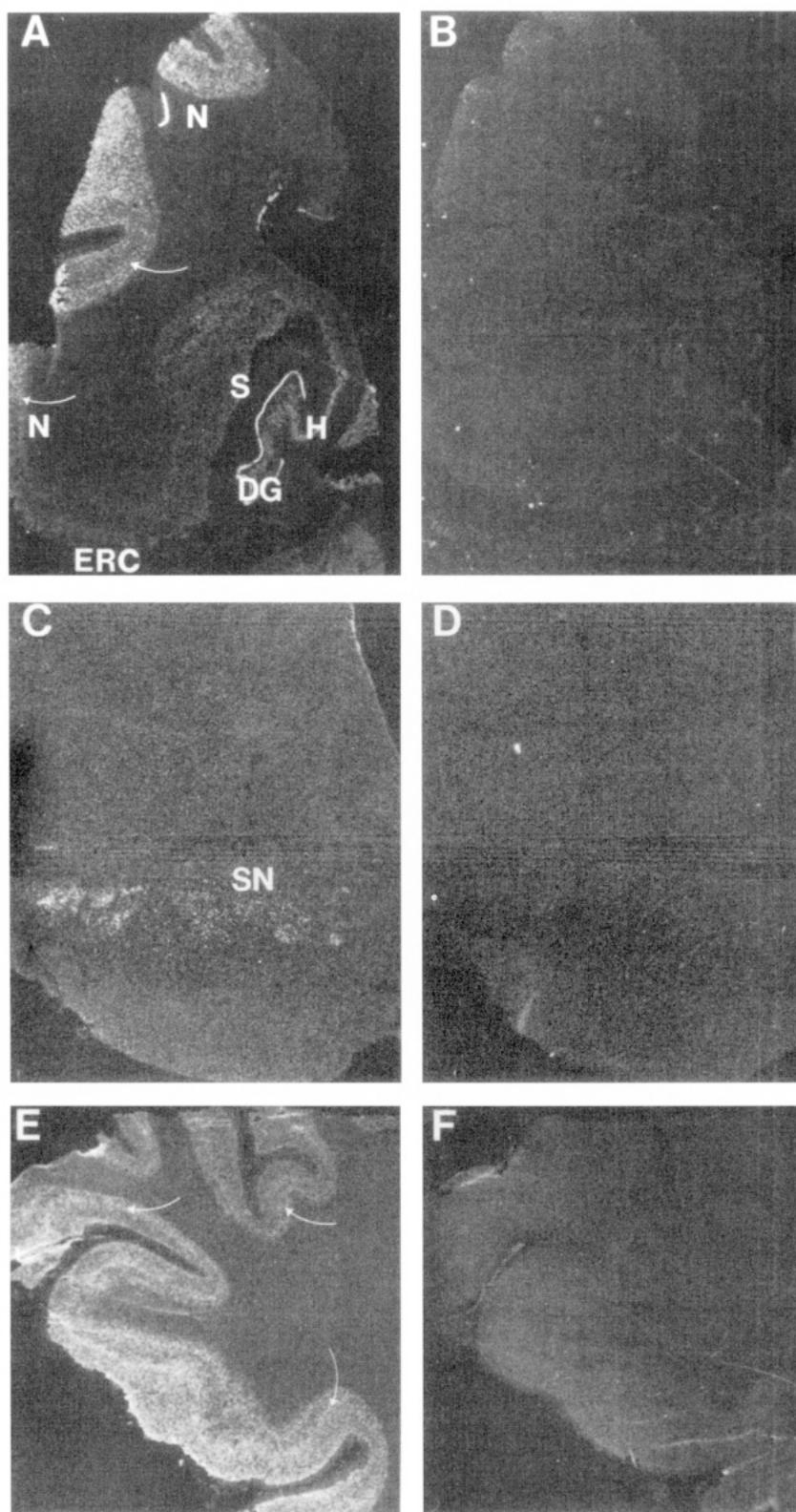


FIGURE 5: *In situ* hybridization of D5 receptor mRNA in human brain. Sections of human brain were incubated with a 5' 35 S-labeled 198 bp riboprobe under conditions described under Materials and Methods. Panels A, C, and E are darkfield micrographs following hybridization with an antisense probe, and panels B, D, and F are after treatment with a sense-strand probe. *Panel A* is at the level of the hippocampus, and is very similar to observations made in the monkey brain. The dentate gyrus (DG) has abundant mRNA, and hippocampal subfields (H) appear to have modest levels of D5 receptor mRNA. Fainter labeling is present in the entorhinal cortex (ERC) and in the subiculum (S). Diffuse labeling is seen throughout the neocortex (N), with some enrichment in deeper laminae (small arrows). *Panel C* is at the level of the midbrain, and shows some labeling in the pars compacta of the substantia nigra (SN), as seen in the monkey. *Panel E* is from frontal lobe, demonstrating labeling throughout much of the neocortex. Again, there is a suggestion of slight enrichment in the deeper layers of cortex (small arrows). No specific labeling is seen in the matched sense-strand control sections (panels B, D, and F).

receptor subtypes. Unlike D1, the D5 receptor may play no part in coordinating motor function. The presence of D5

message in the hippocampus and the neocortex supports a role in learning, memory, and cognition (Fremeau et al.,

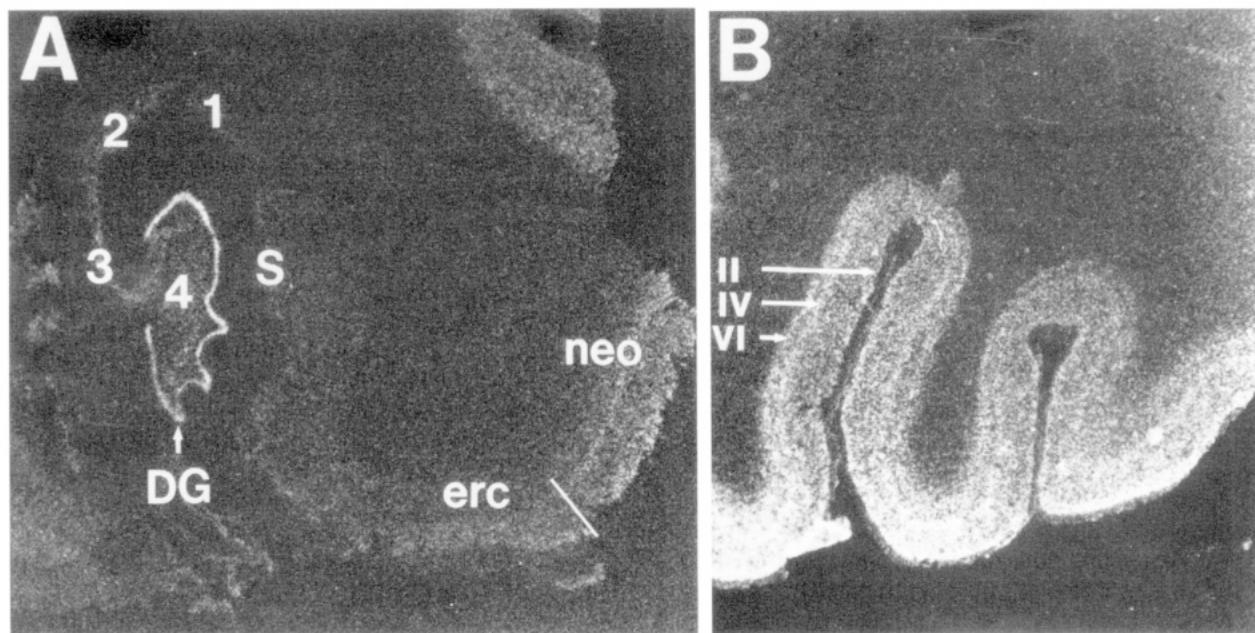


FIGURE 6: Detailed micrographs of *in situ* hybridization of D5 receptor mRNA in human brain. Human brain sections were incubated with a 5'-³⁵S-labeled 194 bp riboprobe under conditions described under Materials and Methods. *Panel A* is at the level of the hippocampus and reveals intense labeling in the granular cell layer of the dentate gyrus (DG). Faint labeling can also be seen in the pyramidal cell layer of CA2, CA3, and CA4 (2, 3, and 4). Somewhat lower levels can be seen in CA1 (1), the subiculum (S), entorhinal cortex (erc), and the neocortex (neo). *Panel B* provides more detail of the labeling seen in neocortex, with the highest D5 receptor mRNAs levels seen in layers II, IV, and VI.

1991; Sawaguchi et al., 1991). Whether the D5 receptor protein exists in these cell bodies or in their axonal or dendritic terminals has yet to be established. The scarcity of the D5 mRNA in human brain slices is consistent with the weak activity of the promoter mutants demonstrated by the luciferase assays.

In conclusion, we have demonstrated that the human dopamine D5 receptor gene is intronic in nature with a large exon 1, of which two splice forms exist. The distribution of the D5 mRNA is rare and discrete. This distribution seemed to have little overlap with the distribution patterns seen of the other four dopamine receptor RNAs (D1–D4), and seems to be distinct from its rat orthologue, D1b. The promoter region of the D5 gene contains several consensus sequences for various transcription factors. Deletion mutation analysis indicated the possible presence of positive and negative modulatory elements. The characterization of these elements is currently under investigation and should provide valuable information concerning the regulatory control of the dopamine D5 gene.

ACKNOWLEDGMENT

We thank Paul Hamel for the P19EC cells, Ram K. Mishra for the SK-N-SH cells, Harry Elsholtz for the vector PL-(KS)b-LUCnPL, and Hong-Chang Guan and Carla Ulpian for excellent advice and technical assistance. We also thank M. Pataki (coordinator), J. Wherrett (Medical Director), and A. Dukzsta (Executive Director) of the Canadian Brain Tissue Bank (supported by the Ontario Mental Health Foundation and the Medical Research Council of Canada).

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BI942815O