Molecular Cloning and Characterization of the 5'-Flanking
and Promoter Region of the Human Dopamine D5 Receptor Gene

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Pharmacology
University of Toronto

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Doctor of Philosophy 1996

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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). In this thesis it was demonstrated 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 in SK-N-SH and GH3 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 positive modulators between nucleotides -52 to -119 and -120 to -182 upstream from the site of transcription initiation. Furthermore, a negative modulator -251 to -500 nucleotides upstream from the site of transcription initiation was also indicated. Within the positive modulatory region there existed a small dinucleotide repeat, termed (TC)13. Genomic DNAs from 18 unrelated schizophrenic (n=7), Parkinson's diseased (n=2), Huntington's diseased (n=6) and normal healthy individuals (n=3) were analysed using a single-strand conformation polymorphism
SSCP assay. SSCP analysis revealed the existence of two additional alleles, termed (TC)\textsubscript{12} and (TC)\textsubscript{14}. Neither form significantly altered D5 promoter-mediated luciferase activity when compared to that of the wild-type control, suggesting that differences in the number of dinucleotide repeats are not likely of any functional consequence for D5 transactivation. Proteins from GH3 and SK-N-SH cell nuclear extracts were able to bind oligonucleotide probes directed to the positive and negative modulator regions of the D5 promoter in electrophoretic mobility shift experiments. Neither of the Sp1 consensus sequences appeared to bind to nuclear proteins and the involvement of Pit-1 and AP1 are currently under investigation. In addition, in order to detect the expression of functional D5 receptor mRNAs and not those of its expressed pseudogenes, in situ hybridisation 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 labelled 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.
Acknowledgments

I would like to express my deepest and most heart-felt thanks to my supervisors, Drs. Philip Seeman and Hyman B. Niznik for allowing me to realize one of my life goals and for providing excellent training and understanding.

I would also like to thank the members of my Ph.D. supervisory committee, Drs. Brian F. O'Dowd, Dr. Allan B. Okey and Dr. Hubert H.M. Van Tol. Their help, advice and friendship was invaluable.

Furthermore, I would like to extend my thanks to my collaborators at the University of Michigan, Dr. James Meador-Woodruff and Scott Damask. I would also like to take this opportunity to thank my friends in the Department of Pharmacology and the University of Toronto. They are Dr. Jeffrey Weiner, Dr. Rachel Tyndale, Dr. Kim Sugamori, Dr. Paul Fletcher, Dr. Brian Ross, Dr. Denis Grant, Adriano Marchese, Dr. Douglas Pon, Dr. Joseph Torchia, Dr. Joseph Francis, Dr. William Durante, Dr. Roger Sunahara, Steven Harding, Ms. Carla Ulpian, Dr. Hong-Chang Guan and many others I have surely failed to mention.

I am deeply grateful to the Ontario Ministry of Health and the Connaught Foundation for the financial support those institutions provided for me during my training.

Mostly, I would like to thank my family, Mary, Lisa and Victor who put up with me and always believed in me.
**Abbreviations**

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<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>dATP</td>
<td>Dideoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Dideoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>ddNTP</td>
<td>Dideoxynucleoside 5'-triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Dideoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dubelco's modified Eagle's Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>dpm</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>Dideoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GHF-1</td>
<td>Growth hormone factor 1 (Pit-1)</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid response element</td>
</tr>
<tr>
<td>G protein</td>
<td>Guanine nucleotide regulatory protein</td>
</tr>
<tr>
<td>HS</td>
<td>Horse sera</td>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>Mr</td>
<td>Molecular weight (relative molecular mass)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>Pit-1</td>
<td>Pituitary-specific growth factor-1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Sp1</td>
<td>Stimulatory protein 1</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard saline citrate buffer (pH 7.0) containing 150 mM NaCl, 15 mM sodium citrate</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris buffer containing 45 mM Tris-borate, 2 mM EDTA (pH 8.0)</td>
</tr>
<tr>
<td>TE (pH 8.0)</td>
<td>Buffer containing 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<td>UV</td>
<td>Ultraviolet</td>
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I. Introduction

Dopamine receptors are the primary targets for therapeutic agents used to treat neuropsychiatric disorders such as Parkinson's disease and schizophrenia. Interest in dopamine receptors and the genes encoding them was originally stimulated by the observation that clinical potencies of most anti-psychotic drugs correlated well with their affinities for a specific population (D2) of dopamine receptors [Creese et al., 1976; Seeman et al., 1976, 1987]. These observations stimulated an intense effort by researchers to characterize the receptors and mechanisms responsible for dopaminergic activity in both normal and diseased brains. While this body of research might suggest that the original dopamine hypothesis of schizophrenia is untenable, it points to a stronger involvement of dopamine D1-like receptors and systems in the pathogenesis of schizophrenia. A growing body of evidence concerning D1-like mediated events and the pathogenesis of schizophrenia has now become available [for review see Jaskiw and Weinberger, 1992; Kahn and Davis, 1995].

To date, the genes for 5 separate dopamine receptors have been cloned; the D1/D1A and D5/D1B which stimulate adenylyl cyclase; and the D2, D3 and D4 which inhibit adenylyl cyclase [for review see Gingrich and Caron, 1993]. Furthermore, it has been demonstrated that there are two transcriptionally competent pseudogenes of the human D5 receptor gene, termed ψD5-1, ψD5-2, which reside on chromosomes 2 and 1, respectively [Nguyen et al., 1991a]. The D5 receptor, not only mimics D1 function but it displays an almost identical pharmacological profile to that of the D1 receptor, except that it possesses a higher affinity for the endogenous neurotransmitter [Sunahara et al., 1991].

Detailed genetic analysis has failed to provide a link between any of the cloned dopamine receptor genes and schizophrenia or any other psychiatric disorder [Coon et al., 1993; Seeman et al., 1994; Sobell et al., 1995; Sommer et al., 1993]. Indeed, since the original report of the cloning of the human dopamine D5 receptor gene [Sunahara et al., 1991], the number of reports concerning the characterization of this gene and its product
have been scant. Information concerning the signals that govern D5 gene expression is not available. However, the possibility that the D5 gene product plays a crucial role in the maintenance of dopaminergic systems and that it may be a critical component in the pathogenesis of psychomotor disorders is more evident than ever before.

This thesis concerns the molecular cloning and characterization of the 5'-flanking and promoter region of the human dopamine D5 receptor gene and the elucidation of its mRNA regional localization within the human and old world monkey brain.

1. Dopamine - An Historical Perspective
   
i.) Dopaminergic Systems - Dopamine, a catecholamine, functions as one of the major neurotransmitters in both vertebrate and invertebrate nervous systems. It elicits its effects by interacting with a select group of cell surface receptors that, in turn are activated and interact with various cellular proteins that lead to a cascade of subsequent effects within the cell (Figure 1). In most animals, dopamine modulates an extremely diverse group of processes including motor control, cognition, emotion, arousal, feeding, neuroendocrine regulation, renal function, and cardiovascular regulation. Furthermore, dopaminergic systems have been implicated in the pathophysiology of several diseases and disorders such as schizophrenia, Parkinson's disease, Tourette's syndrome, and hyperprolactinemia.

   There are four major dopaminergic systems in the mammalian brain. The tuberoinfundibular system projects from diencephalic regions to the hypothalamus where it regulates the secretion of various neuropeptides such as prolactin, β-endorphin and α-melanocyte stimulating hormone (MSH). The incertohypothalamic system projects from the zona incerta to the medial preoptic area and the paraventricular nucleus. The functions of dopaminergic projections in this area are largely unknown and are the subject of a great deal of investigation [for review see Moore and Lookingland, 1995]. The two systems that are thought to modulate an individual's behaviour and motor function and whose
Intracellular Matrix

ATP

cAMP

PKA

gene induction

cFOS cJUN

Figure 1. A schematic illustration of D1-like and D2-like receptor-mediated events within the cell. D1 and D2 receptors interact directly with Gs, Go1f, and Gi, respectively, to modulate adenylyl cyclase activity which initiates a cascade of cellular events. Effects on adenylyl cyclase are dependent on ligand-mediated dissociation of the G-protein-dopamine receptor complex. Activation of cyclase (D1-mediated) leads to an increase in [cAMP], thereby activating cAMP-dependent protein kinase(s) [PKA]. PKA is responsible for the activation of other cellular factors which mediate gene transcription and can result in the activation of immediate-early genes such as c-fos and c-jun.
dysfunction is thought to contribute to various psychomotor disorders are the nigrostriatal and mesocorticolimbic dopamine systems (see Figure 2). Dopaminergic projections in the nigrostriatal system originate in the pars compacta of the substantia nigra and project to the caudate and putamen, among other areas of the striatum. Dopaminergic activity in this region of the brain is thought to be responsible for motor control and degeneration of these inputs leads to Parkinson's disease [for review see Korczyn, 1995]. In the mesocorticolimbic system, the primary dopaminergic neurons originate in the ventral tegmental area (VTA) and project to cortical areas, such as the prefrontal cortex (PFC), as well as projecting to the limbic system, such as the nucleus accumbens, amygdala, hippocampus, septum, olfactory bulb, and the stria terminalis [for review see Roth and Elsworth, 1995]. These dopaminergic systems are thought to be responsible for cognition, learning and memory, as well as motivated behaviours and reward, the dysfunctions of which are thought to be critical components of the pathophysiology of schizophrenia [Williams and Goldman-Rakic, 1995].

**ii.)  **Dopamine Receptor Function

**a.)  **D1-like Receptor Population:  With the use of radioligand binding techniques, it appeared that the D1 receptor population 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]. It is believed that dopamine elicits its effects in these regions through interactions with specific cell surface receptors located both pre- and postsynaptically.

Pharmacological and biochemical studies in the 1970's and 80's provided evidence for two distinct populations of dopamine receptors. The first type to be characterized was the D1 subtype of dopamine receptor that stimulated adenylyl cyclase [Kebabian and Greengard, 1971; Brown and Mackman, 1972; Kebabian et al., 1972; Clement-Cormier et
Figure 2. A simplified schematic representation of the nigrostriatal and mesocorticolimbic dopaminergic systems. Dopaminergic innervation originating from the pars compacta of the substantia nigra (SN) is thought to stimulate movement via the activation of neurons expressing D1-like receptors, dynorphin (dyn) and substance P (sub P), and by the inactivation of neurons expressing D2 like receptors in the basal ganglia. Dopaminergic fibres originating from the ventral tegmental area (VTA) innervate limbic and cortical areas and are thought to influence behavioural parameters such as arousal, cognition, learning, memory, and reward.
This subtype had a high affinity for benzazepines such as SKF 38393, a selective D1 agonist. Table 1 outlines some important characteristics of dopamine receptor subtype classification.

The existence of D1-like receptor subtypes has also been reported in several peripheral regions such as the retina [Lankford et al., 1988], parathyroid gland [Niznik et al., 1988; Niznik et al., 1989; Brown et al., 1989], kidney [Felder et al., 1989b; 1989c], heart [Sandrini et al., 1984] and the pituitary gland [Schoors et al., 1991]. The D1 receptor populations in the heart, retina and parathyroid gland do not appear or have not been shown to be linked to the stimulation of adenylate cyclase [De Keyser et al., 1988; Schoors et al., 1991]. While it has not been determined if D1 receptors couple to adenylyl cyclase in the proximal convoluted tubule (PCT) of the kidney, it has been shown that dopamine stimulation in the PCT inhibits Na⁺-K⁺-ATPase activity via stimulation of adenylate cyclase [Felder et al., 1989c; Bertorello, and Aperia, 1990; Bertorello et al., 1990]. Furthermore, there is evidence that D1-like receptor populations in the kidney stimulated phospholipase C activity in the PCT [Felder et al., 1989b].

The prototypical D1 receptor-containing tissue is the parathyroid gland, where dopaminergic stimulation mediates the release of parathyroid hormone [for review see Civelli et al., 1991]. In the central nervous system (CNS), the D1 receptor mediates increases in c-fos immunoreactivity [Robertson et al., 1992], presumably via stimulation of adenylyl cyclase [Morelli et al., 1992]. The D1 receptor mRNA has been found to be most abundant in the caudate-putamen, nucleus accumbens, and olfactory tubercle, with moderate levels in frontal cortex, hypothalamus, and thalamus [Sunahara et al., 1990; Meador-Woodruff et al., 1991; Mengod et al. 1991; Weiner et al., 1991]. D1 receptors in the striatum modulate the production of dynorphin and substance P, and the degeneration of dopaminergic neurons to this area leads to Parkinson's disease. More recently, the presence of D1/D1α receptor mRNA was detected in the PFC [Yamaguchi et al., 1992]. In the PFC, D1-like receptor activity has been implicated in the maintenance of working
<table>
<thead>
<tr>
<th>Receptor name</th>
<th>D1-like</th>
<th>D2-like</th>
<th>D3-like</th>
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<td><strong>Selective agonists</strong></td>
<td></td>
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<td>SKF38393</td>
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<td>Adenylate cyclase</td>
<td>Stimulates</td>
<td>Stimulation</td>
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<td>PI turnover</td>
<td>Inhibits</td>
<td>?</td>
<td>?</td>
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<td>477</td>
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<tr>
<td></td>
<td>444 (long)</td>
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<td>2.5</td>
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<tr>
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<td>?</td>
<td>7 Introns</td>
<td>5 Introns</td>
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References within text

1 Phosphotidylinositol

Table 1. A summary of the properties of the 5 cloned human dopamine receptor subtypes.
memory. Ionophoretic application of high doses of D1 receptor antagonists to the prefrontal cortex of monkeys reduces their performance in the delayed response task, a condition that improves with dopamine administration [Sawaguchi and Goldman-Rakic, 1991]. However, a paradoxical increase in performance is seen after the application of low doses of D1 antagonists [Williams and Goldman-Rakic, 1995].

b.) **D2-like Receptor Populations:** The second class of dopamine receptor to be identified was the D2 receptor subtype which inhibits adenylyl cyclase [Kebabian and Calne, 1979; Stoof and Kebabian, 1984; Onali et al., 1985]. More recent studies suggest that the D2 receptor also stimulates phosphoinosotide metabolism via a direct activation of phospholipase C [Albert et al., 1990] and may activate K+ channels [Vallar et al., 1990]. The D2 receptor possesses a high affinity for butyrophenone compounds such as spiperone and haloperidol [Seeman, 1987]. The D2 receptor mRNA distribution is similar to that of the D1 receptor being high in mesencephalic cell bodies and, like D1, is also found peripherally in the kidney [Fremeau et al., 1991; Huo et al., 1991; Weiner et al., 1991]. Dopaminergic cells in the mesencephalon thought to express the D2 receptor project to the striatal, limbic and cortical regions. Dopaminergic cells projecting from the hypothalamus to the pituitary are also rich in D2 mRNA [Autelitano et al., 1989].

c.) **Receptor Subtype Localization** - While D2 dopamine receptors are generally believed to exist at both pre- and post-synaptic sites, the D1 or D1-like receptors have long been considered to be post-synaptic [for review see Clark and White, 1987]. This belief is generally based on the ability of autoreceptors to modulate the release of their endogenous neurotransmitter. There is a wealth of electrophysiological, biochemical, and behavioural evidence concerning the subcellular localization of the D2 receptor [for review see Mansour and Watson; 1995; Roth and Elsworth, 1995]. For the D1 receptor, however, the picture is much less clear. The D1-selective partial agonist SKF 38393 does
not seem to affect DA release [Setler et al., 1978]. Furthermore, SCH 23390 (a selective D1 antagonist) failed to reverse the inhibition of DA release in striatal slices by apomorphine, while selective D2 antagonists were effective in blocking these effects [Hyttel, 1984; Lehmann et al., 1983]. In addition, SKF 38393 failed to alter neuronal activity in dopaminergic A10 cells in vivo in electrophysiological studies [White and Wang, 1984], while D2-selective agonists such as quinpirole and pergolide are able to elicit these effects [Brown et al., 1985; Hyttel, 1984; Lehmann et al., 1983; Stoof et al., 1982; White and Wang, 1984]. Moreover, receptor autoradiographic data have demonstrated SCH-23390 binding in the substantia nigra, but there is a lack of correspondence with mRNA signals as revealed by in situ hybridization studies [Mansour et al., 1991]. Mansour and Watson [1995] argue that these receptors originate from striatal neurons and are transported retrogradely to the substantia nigra and, therefore, are not autoreceptors. This seems to be a plausible argument in regard to the fact that there is also no D1 message in the ventral tegmental area. These areas are the origin of the majority of dopaminergic cells in the nigrostriatal and mesocorticolimbic systems. Taken together, these data tend to suggest that D1 dopamine receptors do not function as autoreceptors. However, others have suggested that the lack of correspondence between [3H]-SCH23390 binding and D1 receptor mRNA signals in the substantia nigra may reflect that these D1 receptors are on afferent projections and therefore pre-synaptic [Arnt, 1987; Sunahara, 1993]. The lack of D1-specific or D5-specific ligands further complicates the picture in that the two receptor sub-classes cannot be resolved pharmacologically. The lack of specific ligands makes it impossible to discern in autoradiographic binding studies which receptor population is being visualized. Furthermore, the existence of 2 D5 pseudogenes that are transcriptionally competent impedes efforts to resolve D5 messenger RNA's (mRNA) regional localization within the CNS.
ii.)  **D1-D2 Receptor Interactions - In situ** hybridization analyses of D1 and D2 receptor mRNAs have suggested that both receptors may be co-expressed in the individual neurons [Weiner et al., 1991; Mansour et al., 1991]. Furthermore, several behavioural, biochemical, and pharmacologic studies have revealed that D1 and D2 may interact in either an antagonistic or synergistic fashion [for review see Clark and White, 1987]. Most studies have been unable to implicate the specific dopamine receptors subtypes (i.e. D1-D5) involved in these phenomena, but studies performed in homogeneous cell populations expressing both D1 and D2 receptors have demonstrated synergistic effects on arachidonic acid release [Piomelli et al., 1991] and on dopamine-stimulated Na⁺,K⁺-ATPase activity [Bertorello et al., 1990]. The use of isolated and homogeneous cell populations, while inconclusive, also suggest an intracellular, as opposed to intercellular, mechanism of interaction. In vitro studies have also shown a putative D1-like/D2-like link. D1-specific antagonists were able to modulate the binding of D2-specific agonists in post-mortem brain homogenates in radioligand binding assays [Seeman et al., 1989]. The mechanism(s) of these interactions is not known, but it has been suggested that D1 and D2 receptor populations co-existing in the same cell 'share' the βγ-subunit of the heterotrimeric guanine nucleotide binding protein (G-protein) [Seeman et al., 1994]. The shuttling back and forth of the βγ-subunit from D1 to D2 is thought to represent a mechanism by which occupation of βγ by one receptor subtype keeps the other receptor subtype in a state that has low affinity for dopamine (i.e. uncoupled), effectively desensitizing the receptor. Still, the existence of this type of interaction between the two receptor sub-classes may give new insight into the pathogenesis of neuropsychiatric disorders.
2. Cloning and Gene Structure

The advent of molecular biological and gene cloning technology, allowed investigators to identify several different receptor subtypes beyond the traditional D1-D2 classification. To date, five distinct genes have been isolated which encode for members of the mammalian dopamine receptor family. These genes are distinguished by their respective nucleotide sequences, chromosomal localizations and their pharmacology and function after expression in various bacterial and mammalian systems. These receptors have been designated D1/D1A, D2\textsubscript{L}, D2\textsubscript{S}, D3, D4, and D5/D1B [Dearry et al., 1990; Sunahara et al., 1990; Zhou et al., 1990; Bunzow et al., 1988; Giros et al., 1989; Monsma et al., 1989; Chio et al., 1990; Sokoloff et al., 1990; Van Tol et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991]. Like all receptors in the G-protein linked family, their deduced amino acid sequence suggests the existence of 7 hydrophobic regions, likely transmembrane domains, with an extracellular amino terminus and an intracellular carboxy terminus (Figure 3). 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; Lee et al., 1978b]. The D5 receptor gene encodes for a protein consisting of 477 amino acids with an estimated molecular weight (Mr) of 53 000 Da [Grandy et al., 1991; Sunahara et al., 1991; Weinshank et al., 1991]. It has also been shown that the human D5 gene resides on the short arm of chromosome 4 telomeric to the Huntington's disease gene [Eubanks et al., 1991; Grandy et al., 1992; Polymeropoulos et al., 1991], while the two pseudogenes have separate locations on chromosomes 1 and 2 [Grandy et al., 1992].

Expression studies using the cloned dopamine D5 receptor displayed a pharmacological profile similar to that of the D1 receptor, except that it exhibited a ten-fold higher affinity for dopamine [Sunahara et al., 1991]. Figure 4 compares the apparent inhibitory constants (Ki) of several D1-like agonists and antagonists for the D1 and D5
Figure 3. **Putative topology of the dopamine D5 receptor.** The amino acid sequence is represented as a polypeptide chain that transverses the membrane seven times. Also shown are putative N-linked glycosylation sites. This model is based on Strader's interpretation of the β2-adrenergic receptor (Strader et al., 1989).
Figure 4. Comparison of inhibitory constants (Ki) of various D1 agonist and antagonists for the cloned D1 and D5 receptors. A comparison of inhibitory constants obtained for various dopaminergic agonists and antagonists after transfection of COS-7 cells with either D1 or D5 gene-expression plasmid chimeras. Only the low affinity values are used for agonists. Dopamine is highlighted, as its affinity for the D1 receptor is 10-fold lower than that for the D5 receptor [adapted from Sunahara et al., 1991].
receptors. Quite predictably, the D1 and D5 receptors also share a similar gene structure. Genomic and cDNA sequence information has revealed that both receptors are intronless in their coding regions and show a putative amino acid sequence homology of 80% [Sunahara et al., 1991]. The lack of D5-specific ligands makes it impossible, at present, to distinguish both the pharmacological and functional correlates of D5 receptor stimulation from that of the D1 receptor in vivo. For the sake of brevity, apparent D1 and/or D5 receptor function and pharmacology will be referred to as 'D1' or 'D1-like', since these two proteins seem to be pharmacologically indistinguishable at present. 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 pseudogenes could potentially frustrate attempts to determine both the quantification and tissue-specific distribution of D5 dopamine receptor mRNAs in humans.

3. Dopamine and Schizophrenia

i.) The Classical Dopamine Hypothesis of Schizophrenia - During the past two decades a considerable amount of effort has been dedicated to the characterization and understanding of how the neurotransmitter dopamine elicits its effects in the brain. There have been several reports of dopaminergic dysfunction in the brains of psychotic individuals. The primary impetus for this interest in dopaminergic systems was the fact that most clinically effective anti-psychotics (neuroleptics) were DA antagonists whose primary site of action was believed to be at the D2 receptor. In fact, the clinical potencies of most neuroleptics correlated well with their ability to block D2 receptor antagonists [Creese et al., 1976; Seeman et al., 1976]. Figure 5 presents a graphical representation of the correlation between effective neuroleptic plasma water levels in schizophrenic patients and the dissociation constants (Kd) at the D2 receptor. Furthermore, neuroleptics induce catalepsy and block hallucinations caused by dopaminergic compounds [Seeman, 1987].
Figure 5. Correlation of the clinically effective neuroleptic dose with the dissociation constant (Kd) for the dopamine D2-like receptor population. Antipsychotic drugs exhibit potencies or dissociation constants that correlate with their effective plasma water concentrations that block psychotic symptoms in schizophrenic patients. The Kd for clozapine correlates with its plasma water concentration when it is measured at the D4 receptor (drawing by Philip Seeman).
Radioligand binding studies using post mortem brain samples and positron emission tomography (PET) studies have demonstrated elevated levels of D2-like dopamine receptors in the brains of both drug-naive schizophrenic patients and those who had previously received neuroleptic drug therapies [Seeman, 1987; Seeman, 1992; Wong et al., 1986]. Hyperactivity in D2-like systems and a putative link to the pathophysiology of schizophrenia is further supported by evidence suggesting elevated D4 receptors in post-mortem brain tissue samples of schizophrenics [Seeman et al., 1994]. Finally, D1-D2 receptor interactions seem to be absent or decreased in post-mortem tissue samples of schizophrenic individuals [Seeman et al., 1989].

**ii.) A Neo-classical Perspective of Schizophrenia** - Pharmacological profiles and clinical response of neuroleptics point to hyperdopaminergic function as the root cause of psychosis. However, while psychosis seems to be responsive to neuroleptic treatment in a large number of schizophrenics, decreased social interaction, apathy, avolition and decreased cognitive function seem to be refractory to these types of therapies, to a large degree. The fact that psychotic symptoms are relieved, but these so-called 'negative' symptoms remain, has led many to believe that negative symptoms reflect the true pathology seen in many chronic schizophrenics [Andreasen and Olsen, 1982]. Primate studies have suggested that decreased cognitive function, and poor social skills are the result of frontal cortical dysfunction [Myers et al., 1973; Sawaguchi and Goldman-Rakic, 1991; Wilson et al., 1993]. Furthermore, single photon emission computerized tomography (SPECT) and PET have demonstrated that schizophrenics who display negative symptoms had decreased activation of the PFC while performing cognitive tests [Andreason et al., 1992], and decreased blood flow in frontal cortical areas [Wolkin et al., 1992].

There is now a growing body of evidence that suggests cortical dysfunction in schizophrenia involves the mesocortical dopaminergic system. Mesocortical dopaminergic
terminals are primarily of the D1-like receptor subtype as identified by pharmacologic means [Bannan and Roth, 1983], and neuroleptic-naive schizophrenic patients have normal or lower levels of the dopamine metabolite, homovanillic acid (HVA). Furthermore, the extent of decrease in CSF HVA concentrations corresponded to the degree of cortical atrophy in these patients [Widerlov, 1988; Pickar et al., 1990]. While the aforementioned evidence is indirect, animal studies tend to suggest a similar involvement of dopaminergic systems in cortical function. Poorer performance of the spatial delayed-response task (a measure of cognitive ability) was observed in monkeys with lesioning of their dopaminergic mesocortical neurons than in monkeys who were not subjected to lesions [Brozoski, et al., 1979]. Furthermore, D1 receptor antagonists applied iontophoretically produced similar results in monkeys, while DA applications improved performance [Sawaguchi and Goldman-Rakic, 1991]. These data tend to suggest that the mesocortical D1-like receptor system is not only important for working memory, but that hypofunction of this system may lead to the cognitive deficits in schizophrenia.

It also seems that there may be a reciprocal link between mesocortical dopaminergic function and nigrostriatal dopaminergic function. The lesioning of dopaminergic neurons in the prefrontal cortex with 6-hydroxydopamine (6-OHDA) increases dopamine and HVA levels in the striatum [Pycock et al., 1980; Haroutunian et al., 1988]. Furthermore, increasing prefrontal cortical dopaminergic activity with apomorphine reduced dopamine and HVA levels in the caudate nucleus [Jaskiw et al., 1991]. These data, though indirect, suggest a direct modulatory effect on nigrostriatal dopamine systems by the mesocorticolimbic dopaminergic pathway.

There is other evidence that D1-like receptor mediated systems affect D2-like receptor mediated systems. D1-specific antagonists appear to have the ability to reverse or prevent the expression of the extrapyramidal side-effects induced by haloperidol treatment in monkeys [McHugh and Coffin, 1991]. Furthermore, the putative link between D1-like and D2-like populations of dopamine receptors, as demonstrated using radioligand binding
techniques, appears to be absent in schizophrenics [Seeman et al., 1989]. This may suggest that altered D2 receptor function and densities is a result of impaired D1-like receptor function. Again, the identification and relative contribution of any given receptor subtype in this phenomena has not been ascertained.

iii.) Genetics of the Dopamine Receptor Gene Family - Genetic factors may play an important role in the pathogenesis of schizophrenia as determined by family and twin studies [Gottesman, 1991; Bassett, 1992]. With the identification of 5 separate dopamine receptor genes, it was possible for investigators to study the possible role of these dopamine receptor genes in the etiology of schizophrenia. Using genetic analysis, point mutations in the coding regions of various dopamine receptors leading to amino acid changes have been identified [Seeman et al., 1994; Sobell et al., 1994; 1995]. For example, Sobell and colleagues identified a point mutation that caused a premature translation stop signal in the human D5 receptor, presumably leading to the generation of truncated protein in an apparently healthy individual [Sobell et al., 1995]. In each case the affected individuals seemed to be healthy. Several other studies have attempted to identify a possible link between the 5 cloned dopamine receptor genes and schizophrenia, and in each case no link was found [Moises et al., 1991; Seeman et al., 1994; Sherrington et al., 1993; Sobell et al., 1994; 1995]. The negative linkage results may suggest that either the dopamine receptor genes are not defective, or that any defect in these receptor genes does not contribute to the pathogenesis of schizophrenia, or may reflect technological limitations. Schizophrenia is most likely a disorder of polygenic phenotype, and if the penetrance of any one gene is too low, conventional methods may lack the power to resolve the contribution of any one allele of a given gene.
4. Gene Transcription Regulation

i.) Transcriptional Activation - One of the principle mechanisms by which proteins are regulated and controlled is at the level of gene transcription. Eukaryotic genes that encode for proteins are transcribed by RNA polymerase II (Pol II). Sequences 5' to a eukaryotic gene's transcription initiation site are known to modulate and/or be absolutely required for proper transcription of that gene. Sequences absolutely required for transcription initiation \textit{in vitro} are considered to be part or the core or minimal promoter. Some sequences, while not required for transcriptional initiation, modulate the frequency of initiation by several different mechanisms and are termed enhancers (for positive modulation) or silencers (for negative modulation). Enhancer motifs are quite often the targets of nuclear proteins that infer either tissue-specific or temporal regulation, and are generally considered to be functional regardless of orientation. Enhancers located near the transcriptional initiation site have been termed proximal promoter elements, usually within several hundred basepairs of the transcription initiation site. Sequences distal to the transcription initiation site (i.e several kilobases away) have been termed upstream activator sequences.

The nuclear proteins that recognize these sequences are termed transcription factors. These transcription factors are a diverse class of proteins and when bound to their respective DNA consensus sequences can modify the rate of transcription in several ways. In some cases, transcription factors are thought to interact directly with other transcription factors or RNA Pol II, while others work in a stereospecific fashion by inducing DNA bending to allow other factors to interact with RNA pol II, such as the lymphoid enhancing binding factor I (LEF-1) [Giese et al., 1992]. Some transcription factors are thought to affect chromatin structure by interfering with histone binding and making the transcription initiation site more accessible to RNA Pol II [Okino et al., 1995]. Some transcription factors like Sp1 (stimulating protein 1), are constitutively expressed and are not thought to be inducible [Faisst and Meyer, 1992]. Other transcription factors, such as AP-1 (activator
protein I), Pit-1 (pituitary-specific factor I), and CREB (cAMP responsive element binding protein), are inducible via second messenger systems, either by induction of their own genes transcription or phosphorylation by specific protein kinases [for review see Berk and Schmidt, 1990; Morgan and Curran, 1991; Ingraham et al., 1990; Wegner et al., 1993; Kolb et al., 1993]. Another group of transcription factors are ligand-activated, such as the steroid hormone super-class of receptors and the aryl hydrocarbon receptor (AHR) [see Reichel and Jacob, 1993; Parker, 1993; Hankinson, 1995]. In an inactive state (i.e. unbound by ligand) these factors exist in the cytosol of the cell, and upon binding of endogenous factors and/or xenobiotics or environmental toxicants, are able to be translocated to the nucleus, whereupon they act directly on transcriptional regulatory elements.

At least four proteins (and probably many others) that interact with RNA Pol II have been shown by in vitro reconstitution studies to be essential for transcription initiation. These proteins termed TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, TFIIF, TFI-I and TFIJ, form the pre-initiation complex by; (1) binding a core promoter element (TFIID); (2) or, in the case of TFIIA facilitating the binding of TFIID; or (3) directly binding and thereby, stabilizing RNA Pol II, as is the case for TFIIB and F [for review see McKnight and Tjian, 1986; Maniatis et al., 1987; Conaway and Conaway, 1993]. TFIID, is a multisubunit protein that contains a TATA-box binding protein (TBP) and other polypeptides that are required for activator function. Transcription factors that bind to core or minimal promoter elements protect regions starting several bases upstream to several bases downstream in eukaryotic promoters [Buratowski et al., 1989]. Their association with each other, and/or binding to specific sites, form the initiation complex and allow RNA polymerase II to bind with high affinity and initiate transcription. For TATA box-containing promoters, TFIID in association with TBP recognize the TATA box, followed closely by the binding of TFIIA and TFIIB to separate recognition sites. Once TFIIB has associated with the TFIID-TBP-DNA complex, TFIIF-RNA Pol II to complex to the region
around the transcriptional initiation point. Finally the remaining general transcription factors TFIIE, IIH, and IIJ can associate with the pre-initiation complex [for review see Conaway and Conaway, 1993; Tsai and O'Malley, 1994].

ii.) Eukaryotic Gene Promoters - The core promoters for eukaryotic genes that encode for nuclear RNA contain elements that are able to bind nuclear proteins, which help assemble the pre-initiation complex. These different motifs are distinguishing features of different types of promoters. The most common of these is the TATA box motif (named after its consensus sequence), which is capable of binding the TFIID-associated TBP [for review see Conaway and Conaway, 1993]. Functional TATA boxes usually lie between -15 and -30 bp upstream of the transcription initiation site. Quite often TATA box-containing promoters contain a CAAT box, again named after its consensus sequence, situated approximately 50 bp further upstream of the TATA box. Another type of promoter is one that lacks a TATA box and has a high GC content. This type of promoter can contain multiple consensus sequences for the general transcription factor Sp1 and is characteristic of the type of promoter seen for 'housekeeping' genes. These are genes that are usually constitutively active and/or non-inducible and encode for enzymes that are responsible for providing the general metabolic function of the cell [for review see Mouradian et al., 1994; Dynan, 1986]. Transcription can initiate at a number of tightly clustered nucleotides in these types of promoters. Two other types of promoters are those that may contain a sequence termed an initiator (Inr) sequence instead of or in addition to a TATA box [Smale et al., 1990]. The Inr is pyrimidine-rich, with a weak consensus YYCAYYYYYY [Smale and Baltimore, 1989]. TATA-less promoters with an Inr usually initiate transcription at one specific nucleotide (usually the A in the above Inr consensus sequence). Moreover, it appears that the general transcription factor TFII-I replaces TFIIA in the preinitiation complex in these types of promoters [Mouradian et al., 1994].
iii.) The D1 and D2 Gene Promoters - The 5'-flanking regions of the dopamine D1/D1A and D2 genes have been cloned and their transcription-initiation sites determined [Minowa M., et al., 1992; Minowa T., et al., 1992; Zhou et al., 1992; Valdenaire et al., 1994]. During the course of these investigations it was discovered that the D1 receptor gene, formerly considered to be intronless, possesses a small intron in its 5'-untranslated region, with multiple transcriptional start sites, approximately 1 kilobase (kb) from the start of its coding sequence [Minowa M., et al., 1992]. In contrast, the D2 gene possesses 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 human D2 gene also appears to have multiple transcription start sites [Minowa, M. et al., 1992] and furthermore, it now appears that the rat D2 receptor gene transcription is controlled by at least two distinct promoters [Valdenaire et al., 1994]. 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].

Despite the structural similarities to 'housekeeping' gene promoters, the expression of the D1 and D2 genes are both tissue-specific and under a high degree of transcriptional regulatory control [Mouradian et al., 1994; Minowa M., et al., 1993; Minowa T., et al., 1994]. DNase footprinting and electrophoretic mobility shift assay analysis have revealed the presence of two negative modulatory regions within the D2 receptor gene promoter [Minowa M., et al., 1992, 1993]. While one region does not demonstrate any binding to nuclear factors, the other contains multiple Sp1 sites which interact with multiple factors. In addition to Sp1, the latter region also binds, an as yet unknown, 130 kDa protein that can be competed with Sp1 sequences. These data suggest that the first region is an intrinsic structural control element without the need to bind a trans-acting factor to exert its effect, an observation seen in the didydrofolate reductase gene promoter [Pierce et al., 1992]. Furthermore, the nature of the interactions between the Sp1 sequence and the 130 kDa
protein that does not cross-react with Sp1 antibodies have yet to be elucidated. However, Sp1 is generally considered to act as a positive modulator of gene transcription [Gidoni et al., 1985]. Therefore, activation of Sp1 could serve to induce the transcription of the D2 receptor gene.

Recent studies have also suggested a similarly rigid and complicated set of controls governing the expression of the D1 receptor gene. There appear to be several different proteins binding to two regions within the D1 receptor gene promoter, including AP2 and Sp1, or an antigenically-related protein [Minowa T. et al., 1994]. Again, the exact nature of the interactions between various nuclear proteins and their respective target sequences within the D1 promoter activator regions is unknown. Studies on the rat D1 promoter show that while phorbol esters and dexamethasone, alone or in conjunction, fail to induce D1 promoter-mediated chloramphenicol acetyltransferase (CAT) activity, there is a considerable positive modulatory effect by these agents in the presence of 8-bromo-cAMP [Zhou et al., 1992]. While inconclusive, these data tend to suggest the presence of composite response elements, where one transcription factor is dependent on the binding of a separate factor to another site to elicit its effect.

It is reasonable to assume that all the dopamine receptor genes (including D5) whose expression patterns are restricted to distinct regions and cell types within the CNS and periphery are under a high degree of transcriptional control.
5. **Rationale**

The work presented in this thesis concerns the molecular characterization of D5 receptor gene expression. In order to assess the relative importance of the D5 receptor in neuropsychiatric disorders, it was necessary to determine the molecular mechanisms that govern its expression and its neuroanatomical distribution. The human dopamine D5 receptor gene is a candidate gene for possible involvement in these disorders for several reasons: (a) there is a lack of correspondence between neuroleptic action and alleviation of schizophrenic symptoms; (b) a D1-like receptor has been implicated in cortical dysfunction associated with schizophrenia; and (c) D2 and D4 receptors appear to be elevated in schizophrenic patients. Altered receptor levels may also exist for the D5 receptor. Furthermore, the D5 gene and its product warrant attention because its function has not yet been characterized. Attempts to study the D5 receptor gene product have also been complicated by several factors. The lack of D5- and/or D1-specific ligands, makes it difficult to distinguish the different populations of receptors (hence making it difficult to pinpoint the role of the D1 and/or the D5 receptor in the pathophysiology of various psychomotor diseases). Moreover, the existence of 2 transcriptionally competent pseudogenes have frustrated attempts to localize the expression pattern of this receptor within the CNS.

The basic mechanisms underlying the D5 gene's expression pattern has not been investigated. Elucidation of the transcriptional regulation of this gene is a fundamental step prior to assessing the relative importance of the D5 receptor to the pathophysiology of schizophrenia and other psychomotor disorders. The characterization of promoters and enhancer elements provides information concerning the responsiveness of a gene to such signals as steroid hormones, and second messengers (e.g. Ca++, diacylglycerol and cAMP). Understanding the mechanisms by which these signals regulate the D5 gene expression may lead to therapies based on pharmacological manipulation directed at level of gene expression. Moreover, such investigations could potentially identify transcription
factors that are genetically deficient and thereby leading to altered target gene expression. A genetic mutation in the POU-domain protein Pit-1 causes a form of genetic cretinism arising from the inability of transcription factors to interact properly with enhancer elements in the promoters of thyroid stimulating hormone, growth hormone, and prolactin [Tatsumi et al., 1992]. Finally, mutations in cis-acting DNA elements that interact with transcription factors can also lead to altered gene expression. A single base pair mutation (T to C) in the GATA motif in the Duffy antigen receptor gene promoter impairs erythroid gene expression in Duffy-negative individuals [Tournamille et al., 1995]. This project was designed to address these questions and to provide the ground work for future research to investigate the pattern of expression of the human dopamine D5 receptor gene in neuropsychiatric disorders.
6. **Research Objectives**

The major objective of the project described in this thesis was to clone genomic and complementary DNAs 5' of the D5 receptor gene coding region. Comparison of the 5' termini of the D5 receptor cDNA with genomic 5'-flanking sequence information would allow for the identification of a putative transcription initiation start site(s). With this information it would be possible to take D5 gene promoter sequences and attempt to identify the promoter sequences and trans-activating signals responsible for the cell-specific regulation of the D5 receptor gene.

The second purpose of this project was to obtain, in conjunction with Dr. Brian O'Dowd's laboratory, 5' and 3'-flanking region genomic clones of the D5 receptor gene and its two related pseudogenes. The purpose of this investigation was three-fold. First, identification and analysis of the 5' and 3' points of divergence between D5 and its pseudogenes might identify which pseudogene arose from D5 first. Secondly, this analysis might give clues as to the possible mechanism of replication responsible for the production of the D5 pseudogenes. Lastly, and most importantly, if the pseudogene nucleotide sequences diverged from that of the D5 gene within the transcriptional unit (i.e. the 5' or 3' untranslated region), the non-homologous D5 untranslated cDNA sequences could be used to develop a D5-specific riboprobe to help mapping the regional distribution of the D5 receptor gene transcript in the human brain.
II. MATERIALS

Human λEMBL3 SP6/T7 genomic and λ gt 11 cDNA libraries were obtained from Clontech (Palo Alto, CA). The cloning vector pSP73 and the riboprobe vector pGEM-7Zf(+) were obtained from Promega (Madison, WI). The expression vector pCD-PS was generously provided to our laboratory group by Dr. Tom Stormann of the National Institutes of Health in Bethesda, Maryland. Oligonucleotide primers were synthesized by either the Hospital for Sick Children Biotechnology Service, Toronto, Canada or by General Synthesis and Diagnostics, Toronto, Canada. Sequenase version 2.0 was purchased from United States Biochemical (USB). Taq polymerase, GeneAmp® RNA PCR and Thermostable rTth Reverse Transcription® kits were purchased from Perkin Elmer/Cetus (Foster City, CA). The PCR Optimizer® kit was purchased from InVitrogen (La Jolla, CA). Restriction endonucleases, bacteriophage T4 DNA ligase and kinase, Klenow fragment of DNA polymerase I, terminal deoxynucleotidyl transferase, and Lipofectamine® were purchased from Gibco-Bethesda Research Laboratories (Gibco-BRL). The isotopic reagents $[^{32}aP]dCTP$ (3000 Ci/m mole), $[^{32}yP]dATP$ (3000 Ci/m mole), $[^{32}aS]dATP$ (1000 Ci/m mole) an $[^{3H}]SCH23390$ (70 Ci/m mole) were obtained from Dupont-New England Nuclear (Dupont-NEN).

The luciferase reporter gene vector PL(KS)b-LUCnPL was the generous gift of Dr. Harry Elsholtz of the Dept. of Clinical Biochemistry, University of Toronto. The vector CMV-βgal and the mouse embryonal carcinoma cell line P19EC were generously provided by Dr. Paul Hamel of the Dept. of Cellular and Molecular Pathology at the University of Toronto. The SK-N-SH cell line was the generous gift of Dr. Ram K. Mishra of McMaster University, Hamilton, Ontario. The SK-N-MC cells were graciously supplied by Dr. Hubert H.M. Van Tol of the Depts. of Pharmacology and Psychiatry at the University of Toronto and the Clarke Institute of Psychiatry. GH3 cells were the generous
gift of Dr. Roman Zastawny of the Dept. of Pharmacology, at the University of Toronto. ROS17/2.8 cells were the generous gift of Dr. Jane Mitchell of the Dept. of Pharmacology, at the University of Toronto. COS-7 and NB41A3 cells were purchased from the American Type Tissue Collection (ATCC). Several RNAs and cDNAs derived from immortalized cell lines were the generous gift of Dr. Rachel Tyndale of the Addiction Research Foundation, Toronto, Canada. Oligonucleotides harbouring transcription factor consensus sequences and HeLa cell nuclear extract were purchased from either Promega or Stratagene. Trypsin/EDTA, fetal bovine, donor horse sera and all antibiotics were purchased from Gibco BRL. All culture media were purchased from Media Services at the Medical Sciences Building, University of Toronto. All other reagents used were of molecular biology grade.
III. METHODS

1. Genomic Cloning

i.) Library Screening -- 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) (Acc I-Sac I) 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]. In brief, 500,000 independent clones were plated to a density of 4-5 x 10^4 recombinants/150 mm plate. These clones were allowed to grow in a 37°C incubator for approximately 6-8 hrs. Colonies were lifted off the plates twice using nylon filters (Hybond, Amersham). Filters were left on the plates for 2 min. for the first lift and 6 min. for the duplicated lift. These filters were then denatured on 3mm Whatman paper soaked with a solution of 0.4 N NaOH and 1 M NaCl for 3 min. Filters were then neutralized by transferring them to a second sheet of Whatman paper soaked with a solution of 0.5 M Tris-HCl, pH 7.2, and 1 M NaCl, for 3 min. Finally, filters were transferred to a third sheet of Whatman paper soaked with 10X SSC [25 mM NaCl, 2.5 mM sodium citrate (pH 7.0)] for 6 min. Clones were UV-crosslinked to the filters by exposure to 1200 μjoules of ultra-violet radiation with a Stratalinker™ (Stratagene). Filters were pre-hybridized in a solution of 0.1% glycine, 3X SSC buffer, 100 mM sodium phosphate, pH 6.8, 50% deionized formamide, 1X Denhardt's solution, 10% dextran sulphate, and 150 mg/ml sheared salmon testis DNA for 8 hrs. Filters were then hybridized overnight in a solution of identical composition but with the addition of 32P-dCTP-labeled Acc I-Sac I D5 probe (2 X 10^6 cpm/ml). Filters were rinsed twice in 2X saline sodium citrate (SSC), and 1% sodium dodecyl sulphate (SDS), then washed in an identical solution for 30 min. at 37°C, followed by a 51°C wash for one hour with 0.2X SSC and 0.1% SDS. Filters were then exposed to Kodak X-OMAT film overnight at -70°C with an intensifying screen.
Positively hybridizing plaques were replated, lifted, and screened with the D5 probe as described above, and this procedure was repeated again to ensure that each positively hybridizing plaque represents a single population of an independent clone. Positive plaques were picked and used to infect Y1090 bacterial cells and DNA was isolated by the method of Grossberger [1987]. Inserts were then subcloned into the plasmid vector pSP73 for further subcloning and sequencing.

**ii.) Southern Blot Analysis** - In order to determine which restriction fragments from the positive genomic clone hybridized to Acc I/Sac I probe, Southern blot analysis was performed [Southern, 1975]. DNA fragments from a Xho I digest were separated in a 0.8% agarose gel. These fragments were then transferred to a nylon membrane (Zeta-probe, Bio-Rad) using a vacuum transfer system (Tyler Research Instruments, Edmonton, ALTA) under a negative pressure of -700 mm of H$_2$O for 2 hours. After transfer, DNA was denatured into single-stranded form by the addition of 500 ml of 1.5 M NaCl and 0.5 M NaOH, while maintaining a constant negative pressure. Following denaturation the DNA was neutralized in 500 ml of a solution containing 1 M Tris.HCl (pH 8.0) and 1.5 M NaCl for 1 hour. The gel and blot were then washed in 6X SSC for 1 hour, again maintaining a constant negative pressure. The blot was then removed from the apparatus and the DNA fragments were UV-crosslinked with 0.12 Joules/cm$^2$ of UV radiation in a Stratalinker (Stratagene, La Jolla, CA).

The Southern blot was pre-hybridized overnight at 42°C in a prehybridization buffer consisting of 50% formamide, 0.2% polyvinylpyrrolidone (Mr 40 000), 0.2% Ficoll (Mr 40 000), 0.1% tetrasodium pyrophosphate, 0.05 M Tris.HCl buffer (pH 7.5), 1 M NaCl, 1% SDS and 100 mg/ml of denatured salmon sperm DNA. The blot was hybridized the following day in a hybridization solution identical to that used for pre-hybridization except that it contained the $^{32}$P-radionabeled Acc I-Sac I as probe at a concentration of 2
million cpm/ml. The blot was kept in this solution overnight at 42°C. The blot was washed under high stringency conditions as follows: two rinses with 2X SSC, 1% SDS, two 15 min washes with 2X SSC, 1% SDS at 60°C, and two 15 min washes at 60°C. The blot was then exposed to X-ray film at -70°C with one intensifying screen.

Positively hybridizing clones identified by restriction map and Southern blot analysis were subcloned into pSP73 (Promega, Madison, WI), and sequenced in both directions by the dideoxy chain-termination method described by Sanger et al., [1977] using Sequenase® version 2.0, 7-deaza dGTP sequencing kit (United States Biochemical, Cleveland, Ohio).

2. Complementary DNA Cloning

i.) PCR of cDNA Libraries - Human caudate, putamen and temporal cortex λ gt 11 cDNA libraries (Clontech) were subjected to several rounds of amplification by the polymerase chain reaction in order to get sequence information of the 5' UTR of the human D5 gene. The cDNAs used to construct these libraries were isolated from the selected brain regions of a 20 year old black male who was the victim of a homicide. The brain was put on ice approximately 1 hour after death and remained there until dissection. Upon dissection, brain sections were frozen at -70°C in order to minimize RNA degradation and maximize cDNA insert length. Complementary DNAs were synthesized using oligo(dT) and random primers. The average insert length for the caudate library was 0.9 kb, 1.5 kb for the putamen library and 1.1 kb for the temporal cortex library.

Synthetic oligonucleotide primers complementary to different segments of the D5 gene 5'-flanking genomic sequence and to the amino-terminal portion of the D5 coding region were used in combination with each other or with λ gt 11 5' and 3' vector phage arm primers supplied by the manufacturer (See Figure 6). Briefly, for each reaction, 10^7 λgt 11 phage clones (caudate, putamen or temporal cortex) were amplified using 50 - 100
Figure 6. A visual schematic of the oligonucleotide primers employed to amplify D5 cDNAs from caudate, putamen and temporal cortex cDNA libraries. Oligonucleotide pairs corresponding to different regions of the D5 5'-flanking region, depicted above, were used in PCR reactions to obtain cDNA clones to be used for homology screening of cDNA libraries.
ng of each oligonucleotide pair, 200 μM each of dATP, dCTP, dGTP, and dTTP, 2.5 units of AmpliTaq polymerase (Cetus, Norwalk, CT), 50 mM Tris/HCl, pH 7.4, 150 mM KCl, and 1.5 mM MgCl₂ in a final volume of 100 μl and overlayed with approximately 100 μl of paraffin oil. The samples were denatured at 95°C for 5 min., then incubated at 85°C for 3 min., at which time 2.5 units of AmpliTaq was added to each tube. Reactions were then cycled in a DNA Thermal Cycler™ (Perkin-Elmer/Cetus). For each cycle samples were denatured at 94°C for 60 seconds, annealed at 55°C for 60 seconds and extended at 72°C for 60 seconds. Five μl of each reaction was reamplified under identical conditions as described above and run on an 0.8% agarose gel containing 0.01% ethidium bromide. The DNAs were then transferred to nylon and subjected to Southern blot analysis using an Xho I 3.6 kb genomic fragment Nick translated with ³²P-dCTP (approximately 2 000 000 cpm/ml). Positively hybridizing bands were cut out of a low melt agarose gel, blunt-ended and subcloned into pSP73. Clones were then sequenced in both orientations using T7, Sp6 vector primers and oligonucleotides complementary to the D5 5'-flanking genomic sequence.

ii.) cDNA Library Screening with D5 Receptor Gene 5'-Flanking Sequences - The same human caudate, putamen and temporal cortex λ gt 11 cDNA libraries that were used for PCR, were screened using the Xho I 3.6 kb and putamen 460 bp fragments. Approximately, 1 x 10⁶ independent clones from each cDNA library were plated-out at 5 x 10⁴ plaque-forming units per 150 mm plate and lifted in duplicate on to Hybond® filters. Filters were pre-hybridized and hybridized at 42°C in medium identical to that described in 'genomic DNA cloning' except that they contained either [³²P]-labeled Xho I 3.6 kb or the Put 450 bp probes. Filters were then washed at 20°C for 1 hr with 2X SSC and 1% SDS followed by a 55°C wash for one hour with 0.2X SSC and 0.1% SDS.
Positively hybridizing phage plaques were replated, lifted and probed as described above, and this procedure was performed again to ensure that only pure positively hybridizing plaques would be selected. Purified plaques were amplified and isolated as described in 'genomic DNA cloning'. Large inserts (> 5 kb) were subjected to digestion by various restriction enzymes and analyzed by Southern blot. Smaller fragments were directly subcloned into pSP73, and either sequenced or subjected to Southern blot analysis as described above.

iii.) Isolation of Human Total RNA - Human brain samples were obtained from the Canadian Brain Tissue Bank and all post-mortem times (death to freezing interval) were less than 24 hrs. Total RNA was isolated from human post-mortem cortical brain tissues by the method of Chomczynski and Sacchi [1987]. In brief, approximately 50-100 mg of frozen human cortical brain tissue was homogenized in 2 ml of RNAzol™ B in a glass homogenizing tube using a Teflon pestle. Two hundred μl of chloroform was added to the homogenate and shaken vigorously for 30 secs and then placed on ice for 5 minutes. After incubation, samples were centrifuged at 12,000 X g at 4°C for 15 min in an Eppendorf microcentrifuge. The upper portion of the upper aqueous phase was aspirated carefully to insure that none of the organic phase or interphase was taken up to reduce the likelihood of DNA and/or protein contamination. The aqueous phase was transferred to a new tube, to which was added 2 volumes of 100% ethanol and 0.1 volume of 3M Na-acetate pH 7.5. This mixture was incubated on ice for 15 min and centrifuged at 12,000 X g at 4°C for 15 min. The pellet was washed with 75% ethanol made up with diethylpyrocarbonate (DEPC)-treated RNase-free H2O, and re-centrifuged at 12,000 X g at 4°C for 30 min. The aqueous portion was aspirated and the total RNA pellet was dried by lyophilization and resuspended in 20 - 40 μl of DEPC-treated H2O. Small aliquots of all samples were run in formaldehyde-agarose gels and optical densities were determined at...
260 and 280 nm in a Hitachi U-2000 spectrophotometer. RNA samples were then treated with RNase-free DNase, and either used immediately or stored at -70°C until needed.

iv.) 5' Rapid Amplification of cDNA Ends - 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 (see Section III.2vii). The 5'-RACE reactions were performed using a 5'-RACE System (Gibco/BRL, Gaithersburg, MD) under conditions essentially described by the manufacturer. The 5'-RACE method is illustrated in Figure 7. In brief, less than 1 μg of total RNA was DNased for 1 hr at room temperature and heat inactivated at 90°C for 5 minutes. The RNA was then reversed transcribed with Superscript RT™ at 37°C using a D5-specific primer P1 (5'-GTAGCGGTCCACGCTGATGACGCA-3') as a primer, 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 dCTP. To identify the point of transcription initiation, cDNAs were subjected to two rounds of PCR amplification, using primer P2 (5'-GAAAGGGATGTGACAGCAGCAGCAGCTGCAT-3'), complementary to bases -1363 to -1391, and the manufacturer-provided anchor primer and then reamplified with P3 (5'-CCTCCTCCATCCTGTITGATGTTGGCAGC-3'), complementary to bases -1747 to -1776, and the anchor primer (see Figure 17). In brief, 5-10 μl aliquots of the first-strand synthesis reactions were amplified using 50 - 100 ng of each oligonucleotide pair, 200 μM each of dATP, dCTP, dGTP, and dTTP, 2.5 units of AmpliTaq polymerase (Cetus, Norwalk, CT), 50 mM Tris/HCl, pH 7.4, 150 mM KCl, and 1.5 mM MgCl2 in a final volume of 100 μl. PCR conditions were 95°C for 5 min, 85°C for 2 min, at which time 2.5 units of AmpliTaq were added to each reaction tube. Samples were denatured at 95°C for 30 secs, primer (30 pmol) was annealed at 55°C for 30 secs, and extended at 72°C for 1
Figure 7. A schematic of the 5'-RACE method. Total human RNA is reverse transcribed with Superscript™ reverse transcriptase, followed by RNA template hydrolysis with RNase H and single-stranded DNA's purification. Purified cDNAs are 'tailed' with dCTP and terminal deoxynucleotidyl transferase (TdT). The presence of a poly-cytosine tail in a clone denotes the 5' end of a RNA template.
min for 30 cycles, followed by 10 min at 72°C. After Southern blot analysis, all clones obtained were subcloned into pSP-73 and sequenced in both orientations. Other overlapping cDNAs were obtained in a similar fashion using nested D5-specific oligonucleotides P4 (5'-CATGAACATAGTCATCCGG-3') complementary to bases -1853 to -1833, and P5 (5'-AAGCCGTCTAGGATGAGGTGT-3') complementary to bases -876 to -896 of the 5'-flanking region of the D5 gene (see Figure 17).

v.) RT-PCR of Human Total RNA - 5'-RACE of total RNA from human post-mortem brain tissues failed to provide overlapping clones in the regions between base pair -896 and the coding region of the D5 receptor gene. Therefore, human total RNA (mostly from cortical regions) was subject to RT-PCR using various methods. Again, various oligonucleotide primers were used in different combinations to produce D5 cDNA clones (see Figure 8). Using one such protocol, total human RNA was used to produce cDNAs and amplified with a GeneAmp® RNA PCR Kit (Perkin Elmer/Cetus). In brief, to produce single-stranded cDNAs, less than 1 µg of DNase-treated total RNA was incubated in 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 1 mM each of dATP, dCTP, dGTP and dTTP, 100 pM of a D5-specific oligonucleotide reverse transcription primer, and 10 units of RNase Inhibitor for 2 min. at 42°C at which time 50 units of Murine Leukemia Virus (MuLV) reverse transcriptase was added. All reactions were performed in a final volume of 20 µl. The reverse transcription reactions were then allowed to proceed for a further 15 min at 42°C, then heat inactivated at 99°C for 5 min. followed by 5 min at 5°C.

The first amplification by PCR was carried out by the addition of a solution containing 100 pmol of 'upstream primer', 100 pmol of either the same primer used for first-strand synthesis or a 'nested' 'downstream primer' and concentrations of KCl, Tris-
Figure 8. A visual schematic of the oligonucleotide primers employed to amplify D5 cDNA's from human total post-mortem RNA. Human total RNA was subjected to reverse transcription with either MMLV reverse transcriptase or rTth thermostable reverse transcriptase, followed by PCR amplification with oligonucleotide pairs corresponding to different regions of the D5 5'-flanking region, depicted above.
HCl, and MgCl₂ as described above. Samples were denatured at 95°C for 5 min., then incubated at 85°C for 3 min, at which time 2.5 units of AmpliTaq® polymerase was added to each reaction. Each reaction was subjected to 25 cycles of the following: denaturation at 95°C for 30 secs; followed by an annealing step at 55°C for 30 secs., and lastly polymerase extension at 72°C for 60 secs. All reactions were carried out in a final volume of 100 µl and amplification reactions were achieved using a GeneAmp PCR System 9600 Thermal Cycler (Perkin Elmer/Cetus). Samples were reamplified using 'nested' oligonucleotide primers, and in some cases, with primers identical to those used in the first-round PCR reactions. Five µl of each reaction was re-amplified under identical conditions as described above, and run on an 0.8% agarose gel containing 0.01% ethidium bromide. The PCR products were then transferred to nylon membranes and subjected to Southern blot analysis using end-labeled with γ-³²P-ATP primers which were directed to sequences internal to the various primers sets used for each reaction. Positively hybridizing bands were cut out of a low melt agarose gel, blunt-ended and subcloned into pSP73. Clones were then sequenced in both orientations using T7, Sp6 vector primers and oligonucleotides complementary to the D5 5'-flanking genomic sequence.

vi.) RT-PCR using Thermostable Reverse Transcriptase - In an attempt to reduce the formation of secondary structure due to high 'GC' content in the D5 5'-flanking region, total human RNA was subjected to RT-PCR using thermostable rTth DNA reverse transcriptase (Perkin Elmer/Cetus). Oligonucleotide primer pairs for each reaction are shown in Figure 8. Briefly, for each reaction, less than 1 µg of DNA-free total human RNA (again derived from cortical brain regions) was incubated in 2 mM Tris-HCl, pH 8.0, 10 mM KCl 0.01 mM EDTA, 0.1 mM DTT, 0.05% Tween 20, 5% glycerol, 1 mM MnCl₂, 200 µM each of dATP, dCTP, dGTP, and dTTP, and 100 pM of a D5-specific oligonucleotide reverse transcription primer, for 5 min. at 70°C, followed by the addition
of 5 units of rTth reverse transcriptase to each reaction. Samples were then incubated for another 15 min. at 70°C. Reactions were stopped by placing the tubes on ice. All reactions were carried out in a final volume of 20 μl. The first round PCR reaction was carried out by the addition of an 80 μl solution to each reaction tube that included a D5-specific 'upstream' primer at a final concentration of 100 pM, 2.0 mM MgCl2 and 0.8 X MnCl2 chelating solution. In this protocol, it is necessary to chelate manganese in order for the rTth to have efficient DNA polymerase activity necessary for the PCR reaction. Any subsequent re-amplification reactions and the analysis of positively hybridizing clones were carried out as described in 'RT-PCR of Human Total RNA'.

vii.) Expression in COS-7 Cells - To obtain cDNA clones in the 'GC'-rich region of the 5'-UTR flanking between bases -896 and the coding region of the D5-gene, a 5.7 kb (Eco RI) genomic fragment, encompassing the entire transcriptional unit of the D5 gene, was subcloned into the expression vector pCD-PS [Stormann et al., 1990]. A plasmid map of this vector is presented in Figure 9. The expression vector pCD-PS/D5 gene construct utilized the Simian Virus 40 (SV40) promoter and polyadenylation signals to initiate and terminate transcription, respectively. This construct was transiently transfected into COS-7 cells using a polyamine-mediated transfection (Lipofectamine™, Gibco/BRL) method (see below). The human COS-7 cell line expresses the T-antigen which in turn is capable of enhancing transcription via the SV40 promoter. Total RNA was isolated, as described above, and reverse-transcribed with 50 units of MuLV reverse-transcriptase (GeneAMP® RNA PCR kit; Perkin Elmer/Cetus) and 7-deaza-dGTP:dGTP (3:1), as per manufacturer's instructions (described in 'RT-PCR of Human Total RNA'). The oligonucleotide primer used for reverse transcription was termed P7 and corresponded to the antisense strand of the D5 gene between bases +255 and +232 (5'-CACGGCCAGAGACACGATGAAGAC-3'). Single-stranded DNA was subjected to one
Figure 9. Plasmid map of the D5 genomic EcoR I fragment subcloned into pCD. The EcoR I fragment encompassing the entire 5'-UTR and coding region of the human dopamine D5 receptor gene was placed 3' to the SV40 promoter in the expression vector pCD and transfected into COS-7 cells in order to produce workable amounts of D5 mRNA for use in RT-PCR cloning experiments.
round of PCR, a reaction with 7-deaza-dGTP:dGTP, using oligonucleotide primers P6 (5'-GGTGGTGAAAAAGGACAATCTC-3') complementary to bases -1009 to -986, and P10, complementary to bases +26 to +1; or oligonucleotides P8 (5'-AAGCGTGCCCCAGGGCTAAGG-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.

3. **Regional Distribution of D5 Receptor mRNAs**

i.) **Northern Blot Analysis** - Northern blot analysis was performed using a 'Multiple Tissue Northern (MTN) Blot' (Clontech) with various probes directed to different portions of the human D5 dopamine receptor gene. Each lane contained at least 2 μg of poly-A-RNA extracted from the amygdala, caudate nucleus, corpus callosum, hippocampus, hypothalamus, substantia nigra, subthalamic nucleus and thalamus of a 20 year old healthy black male who died of a gunshot wound. The blot was hybridized with a 1.2 kb Sma I-Sac I restriction endonuclease fragment of the D5 receptor gene coding region which was [³²P]-radiolabelled by nick translation (Amersham). The blot was hybridized for 16 hours in a buffer containing deionised formamide, 1X Denhardt's solution, 10% dextran sulphate and 150 mg/ml sheared and denatured salmon testis DNA. The blot was washed in 2X SSC and 1% SDS for 1 hour at room temperature followed by 2 x 15 min. washes in 0.2X SSC and 1% SDS at 60°C. The blot was exposed to Kodak XAR autoradiographic film with two intensifying screens at -70°C for 7 days. After autoradiography the blot was stripped of probe as per the manufacturer's instructions and reprobed with a [³²P]-labelled 198 bp clone corresponding to the D5 5'-UTR between nucleotides -2022 to -1924, a region that shared no homology with either of D5-like pseudogenes. The blot was probed, washed and exposed to film as described above.
ii.) *In Situ Hybridization of D5 Receptor mRNAs* - *In situ* hybridization studies were performed by Dr. James Meador-Woodruff’s laboratory at the Mental Health Research Institute, University of Michigan in Ann Arbor. 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, tissue blocks were excised, and rapidly frozen on dry ice. These individuals were all male who died suddenly (3 were homicide victims, and one died from an acute myocardial infarction). Postmortem intervals of samples were less than 24 hours. 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 minutes. *In situ* hybridization was performed as previously described [Meador-Woodruff et al., 1991, 1993]. Briefly, sections were hybridized with [35S]-labeled riboprobes (10-20 x 10⁶ dpm/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' end 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 minute wash in 0.5X SSC at 55°C. The slides were then dehydrated and exposed to Kodak X-OMAT film for 4-6 weeks. Control slides, including both “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].
4. Functional Analysis of the D5 Gene Promoter Region

i.) RT/PCR of Mammalian Cell Lines - Total RNA isolated from different cell lines were subjected to RT/PCR in an attempt to identify those that express the D5 gene. First strand cDNA synthesis was performed as described above using oligonucleotides D1b-1 (5'-TCGTCACTCATCAGCTTACATC-3'), complementary to bases 1027 to 1050. Purified single-stranded cDNAs were subjected to PCR using identical conditions as those described in 'cDNA Cloning'. PCR amplifications were carried out using oligonucleotides D1b-1 and D1b-2 (5'-ATTGAGAGAGGAGTTGGCCCAGCC-3'), complementary to bases 667 to 690. These oligonucleotides were directed to homologous coding regions of both the human D5 and rat D1b receptor gene.

ii.) D5-Luciferase Plasmid Constructs and Assays - 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 (Xba I) 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'-ATGCAAGGTCTTTTCTCATAATG-3') (see Figure 22). This fragment was then blunt-ended, followed by restriction digestion at the 5'-end with Xba I, 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 Hind III or Eco RI, respectively, followed by religation. p251D5-LUC was generated by PCR using oligonucleotide primers P11 (5'-ATCCACCCACCTCGGCTCCCCAAA-3') and P10 and p119D5-LUC were created in a similar fashion using oligonucleotides P12 (5'-GGGTAAAACAACATATGGGA-3') and
Figure 10. Plasmid map of the D5 promoter deletion mutants subcloned into the PL(KS)b-LUCnPL reporter gene vector. Promoter deletion mutants containing either 500, 251, 182, 119, or 51 bp of sequence information 5' of the transcriptional start site and 197 bp of sequence information 3' of this site was subcloned upstream of the firefly luciferase gene for use in reporter gene assays to determine the regulatory regions within the D5 promoter.
The D5 promoter sequence information included in these constructs as well as a plasmid map of the PL(KS)b-LUCnPL vector are provided in Figure 10.

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 a-minimal essential medium supplemented with 10% fetal bovine serum. The human pituitary cell line GH3 was maintained in Ham's F-10 with 10% horse serum and 2.5% 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 µg of the heterologous D5-luciferase constructs or luciferase plasmid alone, together with 2 µg of the expression vector CMV-βGAL, were incubated with 80 µg of Lipofectamine™ at room temperature for 45 minutes in 1 ml of sera-free media. Cells were washed with serum-free media and the transfection mixture added to each plate in a total volume of 5 mls. After 5 hrs, cells were washed with phosphate-buffered saline and maintained with the appropriate media supplemented with sera. Twenty-four hrs after transfection, cells were harvested in 300 ml of lysis buffer, containing 50 mM TRIS/2-[N-morpholino]ethanesulfonic acid (MES) pH 7.8, 1mM DTT and 0.1% Triton X-100. Cell lysates were vortexed for 10 secs and briefly spun at 14,000 x g in an Eppendorf 5415 C microcentrifuge to pellet cellular debris. Using a Bio-Orbit 1250 Luminometer, 200 µl of cell lysate together with 15 µl of 750 mM TRIS/MES (pH 7.8), 150 mM MgOAc and 40 mM ATP, was mixed with 200 µl 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.
iii.) \textit{β-Galactosidase Activity in Cell Extracts} - \textit{β}-galactosidase activity determinations were performed essentially as described by Sambrook et al. [1989]. For each determination, 30 µl of cell extract was added to 3 µl of 100 x Mg solution, 66 µl of 1 X \textit{O}-nitrophenyl-\textit{β}-D-galactopyranoside (ONPG), and 201 µl of 0.1 M sodium phosphate, pH 7.5. 100 x Mg solution is 0.1 M MgCl$_2$ and 4.5 M \textit{β}-Mercaptoethanol. 1 x ONPG is a 4 mg/ml solution of ONPG dissolved in 0.1 M sodium phosphate, pH 7.5. 0.1 M sodium phosphate was made by mixing 41 ml of 0.2 M Na$_2$HPO$_4$·2H$_2$O, 9 ml of 0.2 M NaH$_2$PO$_4$·H$_2$O, and 50 ml of H$_2$O. Samples were incubated at 37°C until a faint yellow colour appeared. The reactions were stopped by the addition of 500 µl of 1 M Na$_2$CO$_3$ and optical densities were read at a wavelength of 420 nm in a spectrophotometer.

iv.) \textit{Effect of Forskolin and Phorbol Esters} - In order to determine if the transcription factor AP1 could stimulate D5 promoter-mediated luciferase activity, SK-N-SH and GH3 cells were transfected with 10 µg of either the p500D5-LUC or the p251D5-LUC D5-luciferase chimera plasmids or the promoterless vector PL(KS)b-LUCnPL as described in 'D5 Luciferase Plasmid Constructs and Assays'. Five hours after DNA transfection, the transfection medium was removed and replaced with 10 mls of sera-containing medium (for every 100 mm plate), with 12-O-tetradecanoylphorbol-13-acetate (TPA, 0.1 µM or 1.0 µM), 5.0 µM forskolin, or 1.0 µM TPA and 5.0 µM forskolin. Plates that did not receive TPA or forskolin were treated with vehicle (EtOH, as described above). Never, in any case did the volume of vehicle exceed 0.1% of the final volume of media. Cells were harvested 24 hrs later and assayed as described above.
v.) **Effect of Dexamethasone** - In order to ascertain the effect of glucocorticoid receptor activity on D5 gene transcription, D5 promoter mutant-luciferase chimera containing plasmids were transfected into ROS 17/2.8 rat osteosarcoma cells. The ROS 17/2.8 cells were maintained in Dulbecco's modified Eagle's media (high glucose) with 10% fetal bovine serum. The glucocorticoid receptor is known to be expressed in this cell line [Rodan and Rodan, 1986; Catherwood, 1985]. Transfections were carried out as described in 'D5 Luciferase Plasmid Constructs and Assays' using the D5-Luciferase chimera plasmid p51-D5LUC and the promoterless vector PL(KS)b-LUCnPL. Five hours after DNA transfection, the transfection medium was removed and replaced with 10 mls of sera-containing medium (for every 100 mm plate) and either 0.1, 0.5, or 1.0 μM dexamethasone, according to the method described by Mordacq and Linzer [1989]. Plates that did not receive dexamethasone were treated with vehicle (EtOH, as described above). Cells were harvested 24 hrs later and assayed as described above.

vi.) **Preparation of Nuclear Extract** - HeLa cell nuclear extract was obtained from Stratagene. SK-N-SH and GH3 cells were grown on 100 mm plates to confluency (10⁷ cells) and nuclear extracts were prepared by the method of Schreiber et al. [1987], with slight modifications. Cells were washed twice with PBS and harvested by the addition of 5 ml of 1 x SSC and incubated at 37°C for approximately 1 min. The SSC was then aspirated and cells were washed off the plates with PBS. Cells were then pelleted by centrifugation for 10 min at 2000 rpm in a Beckman GPR centrifuge. Cell pellets were washed with 500 μl PBS/10⁷ cells and resuspended in buffer A (500 μl/10⁷ cells). Buffer A consisted of: 10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 1 mM PMSF. Cells were gently resuspended in a 200 μl pipette tip, transferred to a 1.5 ml microcentrifuge tube and incubated on ice for 15 minutes, after which 30 μl of cold 10% NP-40 was added. Cells were then vortexed vigorously for approximately 10
secs. and then centrifuged at 4°C in an Eppendorf microcentrifuge at 14,000 rpm for approximately 30 secs. The supernatant was discarded and the pellet was resuspended in 50 µl of buffer B. Buffer B consisted of: 20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF. This mixture was then rocked gently for 15 min. at 4°C and then centrifuged at 4°C and 14,000 rpm for 5 min. The supernatant which contained the crude nuclear extract was aliquotted into 30 - 60 µl portions and stored at -80°C.

vii.) Protein Concentration Determination - Protein concentration of the nuclear extracts was determined using a Bio-Rad™ Protein Assay kit (Bio-Rad Laboratories, Hercules, CA.). Assays were performed essentially according to the manufacturer's protocol. In brief, one part of reagent dye was diluted in four parts H₂O and 2.5 ml of this solution was added to either 50 µl of protein sample or standards containing 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml of bovine serum albumin. Samples were incubated for approximately 5 min. at room temperature and colour change was measured in a spectrophotometer at 595 nm. A standard curve was constructed and protein concentrations of the nuclear extract samples were interpolated using this curve.

viii.) Electrophoretic Mobility-Shift Assay - To determine whether the D5 promoter binds nuclear proteins, double-stranded oligonucleotides were synthesized, the sequences of which are depicted in Figure 11.

Complementary single-stranded oligonucleotides were annealed to form double-stranded (ds)-oligonucleotides. To anneal the oligonucleotides, 500 ng of each oligonucleotide resuspended in 5 µl water was mixed with 2 µl of annealing buffer (Pharmacia) in a volume of 20 µl. Oligonucleotides were denatured at 85°C for 2 min, then heated at 65°C for 15 min, followed by 15 min at 37°C and then placed in an ice water bath.
Figure 11. Nucleotide sequences of the sense strand of oligonucleotide probes used in gel retardation experiments

GS-1 (5'-GTGTGGTGCTGATCTGGGACACTGAAAGCTCCTCTGGTTCTCAGCATATTTCCTGCCTTCTGCT-3');
GS-2 (5'-CAGCCTCCCAAGTTAGGGCCACTACAGGCCCCACCATGTCTGCTAACATTTCCTTTTGTTTAGT-3');
GS-3 (5'-GCATCTCTCTCTCTCTCTCTCTCTCATTTGCTATATAA-3');
GS-4 (5'-TGGGGAACATATGGACATGTGCTATTTTTTGCTGAATACAAAGAATGACTG-3').
for 15 min. Oligonucleotides were then either stored at -20°C to be used in competition experiments or were then 'end-labeled' with γ-32P-ATP using T4 polynucleotide kinase (Pharmacia). Double-stranded oligonucleotides were then purified on Nacs Prepac® columns (Life Technologies Inc., Gathersburg, MD).

ix.) EMSA Binding Reaction Conditions - DNA-protein binding reactions for electrophoretic mobility shift assays were carried out with approximately 3 μg of nuclear extract in the presence of 5 μg of poly dI.dC and 10 mM Tris-HCl, pH 8, 40 mM KCl, 6% glycerol, 1 mM DTT and 0.05% NP-40, in a final volume of approximately 23 μl. This reaction mixture was allowed to equilibrate at room temperature for 15 min. At this time approximately 2 μl of probe (< 100,000 cpm) was added to the mixture and allowed to equilibrate at room temperature for a further 15 min. Samples were taken up in 5 μl of 6 x loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol and 15% Ficoll), and immediately loaded onto the gel. Gels of 4-6% acrylamide (99:1 acrylamide/N,N'-methylenebisacrylamide, w/w) were run in 0.5 x TBE (1 x TBE = 0.089 M Tris, 0.089 M boric acid, and 0.002 M EDTA) at 100 V and then dried prior to autoradiography.

For competition experiments, 50 and/or 100-fold molar excess unlabeled competitor ds-oligonucleotide was added to the reaction mixture before the addition of probe. The unlabelled oligonucleotides used in competition reactions are shown in Table 2. They included consensus sequences for the transcription factors AP-1, AP-2, Sp1, CREB, GRE, NF-κB and CTF/NF1 as well as the same oligonucleotides that were used as probes.
<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Consensus Double-Stranded Oligonucleotide</th>
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<tbody>
<tr>
<td>Sp1</td>
<td>5' GATCGATCGGGCGGGCGATC 3'</td>
</tr>
<tr>
<td></td>
<td>3' CTAGCTAGCCCGCCCGCTAG 5'</td>
</tr>
<tr>
<td>AP-1</td>
<td>5' ATTTTGGCTTAAGCCATAATG 3'</td>
</tr>
<tr>
<td></td>
<td>3' TAAAACCGAATTCTGTTTATAC 5'</td>
</tr>
<tr>
<td>AP-2</td>
<td>5' GATCGAAGCTGACCCGCGGCGGCCGCGGT 3'</td>
</tr>
<tr>
<td></td>
<td>3' CTAGCTTTGAGCTGCCCGCGCCCGGCGGCA 5'</td>
</tr>
<tr>
<td>CREB</td>
<td>5' GATTGGCTGACGTAGAGAGCT 3'</td>
</tr>
<tr>
<td></td>
<td>3' CTAAACGACTGAGCTCTCTGCA 5'</td>
</tr>
<tr>
<td>GRE</td>
<td>5' GATCAGAAGACAGATCTCTCTTA 3'</td>
</tr>
<tr>
<td></td>
<td>3' CTAGCTTTGAGCTACAGAGAT 5'</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5' GATCGAGGGAGTTTTCCCTAGC 3'</td>
</tr>
<tr>
<td></td>
<td>3' CTAGCTTCCTGAAAGGATCG 5'</td>
</tr>
<tr>
<td>CTF/NF1</td>
<td>5' ATTTTGGCTTAAGCCATAATG 3'</td>
</tr>
<tr>
<td></td>
<td>3' TAAAACCGAATTCTGTTTATAC 5'</td>
</tr>
</tbody>
</table>

*All oligonucleotides were obtained from Stratagene (La Jolla, CA) for use with their Gelshift™ kit.
SSCP Analysis of the D5 Gene Promoter Region - Genomic DNA was extracted from blood samples (n=14), and in some cases from human postmortem brain (n=4), from 18 unrelated individuals. Seven individuals suffered from schizophrenia, 6 from Huntington's Chorea, and 2 from Parkinson's disease, while 3 other individuals were healthy. Blood samples (collected in vacuum tubes containing ethylenediamine tetra-acetic acid) were obtained from consenting schizophrenics in the Toronto Schizophrenia Registry, from schizophrenic outpatients at Hillside Hospital (Glen Oaks), and from the Allan Memorial Institute (Montreal).

Genomic DNA was extracted from postmortem brain tissue samples [Ausubel et al., 1988] or from blood samples by the method of Miller et al. [1988]. Briefly, 100 mg of tissue or 3 ml of blood were mixed with 9 ml of a solution containing 155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA. After centrifugation at 3 000 x g for 10 min. at 4°C, the remaining tissue was digested for 3 h at 55°C with proteinase K (125 µg/ml) in 3 ml of nucleus-lysis buffer (75 mM NaCl, 25 mM EDTA, 1% SDS). Proteins were precipitated with 1 ml of 6 M NaCl. After centrifugation (3 000 x g for 10 min.), the DNA was precipitated with ethanol, dried, dissolved in 500 µl of buffer (10 mM Tris, 1 mM EDTA; pH 8) and stored at 4°C.

Genomic DNA was amplified using the polymerase chain reaction in vitro. The oligonucleotide primers used to amplify the TC-repeat region were (P1 5'; position -2376) ATCCACCCACCTCGGCCTCCCAAA (3') and (P2 5'; position -2222) GTCCCATATGTGTGGTTTACCACCT (3') (see Figure 12). Each PCR reaction was performed in a 100 µl volume using 100 to 500 ng of genomic DNA, 100 ng of each primer, 200 µM each of dATP, dCTP, dGTP, and dTTP, 2.5 units of AmpliTaq polymerase (Cetus, Norwalk, CT), 5% DMSO and 20 µl of Invitrogen buffer C (Invitrogen Corp., San Diego, CA). PCR conditions were 95°C for 5 min., 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 secs, primer (30 pmol) was annealed at 55°C for 30 secs, and extended at 72°C for 1 min. for 30 cycles, followed by 10 min. at 72°C.

Single-strand conformation polymorphism analysis [modified from Orita et al., 1989] was used to detect possible variations in the nucleotide sequence of the dopamine D5 receptor gene promoter. Two μl of the PCR product were added to tubes containing 13 μl of a mixture of 5 parts of solution X (0.1% SDS and 10 mM EDTA), and 8 parts of solution Y (95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol and 20 mM EDTA). The sample was then heated to 96°C for 5 min., then placed immediately on ice. Five μl of this mixture was used in each lane of a 5% polyacrylamide gel, containing a bisacrylamide/acrylamide ratio of 1:60 in 0.5 X TBE buffer (89 mM Tris base, 89 mM Boric acid, and 2 mM EDTA, pH 8.0). The electrophoresis apparatus used was a Diagen temperature gradient electrophoresis system (TGGE; Diagen GmbH, Dusseldorf, Germany) with 2 Neslab RTE-100 water-circulating coolers (Neslab Instruments, Mississauga, Ontario) both set at 8° C. The buffer chambers of the electrophoresis system were filled with 0.5 X TBE buffer. The gel was electrophoresed at 8° C at a constant voltage of 300 V with 12 mA until the bromphenol blue dye reached the end of the gel. The gel was rinsed twice in 10% ethanol containing 0.5% acetic acid, stained for 10 min. in 0.1% silver nitrate, followed by two rinses in water, and the silver reaction finally developed by rinsing the gel for 20 min. in a freshly prepared solution of buffer C (1.5% NaOH, 0.01% NaBH₄ and 0.15% formaldehyde); the gel was fixed for 5 min. in 0.75% Na₂CO₃ and stored under Saran wrap.
xi.) **Cloning of D5 Promoter Variants by PCR** - A segment of the D5-5'-flanking region, encompassing 251 bases upstream (5') of the transcription initiation site, and 201 bases downstream (3') of this position was generated by PCR of genomic DNAs extracted from the tissues of the individuals who displayed variant banding patterns, using oligonucleotide primers P1 and another primer, P3 (P3 5'; position -1928) ATGCAAGGTCTTTTCCCTATATTG (3'). The primers used to amplify regions of the D5 promoter for SSCP analysis and cloning are illustrated in Figure 12. This fragment was then blunt-ended and subcloned into the luciferase gene-containing plasmid PL(KS)b-LUCnPL to create the D5 luciferase mutants TC12LUC and TC14LUC. Clones were sequenced in both directions by the dideoxy chain-termination method described by Sanger et al., [1977] using a Sequenase version 2.0, 7-deaza dGTP sequencing kit (United States Biochemical, Cleveland, Ohio).

xii.) **D5 Promoter Luciferase Assays** - Relative luciferase activity of the common and variant forms of the human D5 receptor gene promoter/luciferase chimeras were determined in the human neuroblastoma SK-N-SH cell line. The D5 and variant D5 promoter-luciferase chimera plasmids p251-D5LUC (wild-type), 'TC'12 and 'TC'14 were transfected, and luciferase and β-galactosidase activities were determined as described in 'D5-Luciferase Plasmid Constructs and Assay'.
Figure 12. Nucleotide sequence of the human D5 receptor gene promoter region. Arrows P1 and P2 indicate oligonucleotide primers used in the PCR for SSCP analysis. Arrows P1 and P3 indicate the oligonucleotide primers used in PCR to generate clones fused into the vector PL(KS)b-LUCnPL for sequencing and for transient transfections and luciferase assays. Bold type is used for the dinucleotide repeat region.
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IV. RESULTS

1. Genomic and cDNA Cloning

i.) Human Genomic Library Screening - In order to identify the transcriptional start site of the human 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 (Acc I-Sac I) 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. Southern blot analysis revealed that one of these clones contained 3.6 kb and 5.0 kb Xho I fragments (Figure 13). The 5.0 kb fragment contained the D5 coding region and 115 bp of 5' sequence information. The 3.6 kb fragment corresponded to the 5'-untranslated region of the dopamine D5 receptor gene. The two higher bands (9.0-12.0 kb) visualized by ethidium bromide staining later proved to be 3' to the D5 coding region. The Xho I 3.6 kb fragment (Figure 13) was subcloned into pSP73 and from there smaller fragments obtained after digestion with various restriction endonucleases were again subcloned into pSP73 to facilitate sequence analysis. All fragments were sequenced entirely in both directions.

Figure 14, part 'A', provides a restriction map of the 5'-flanking region of the human dopamine D5 receptor gene. Sequence data for the human genomic D5 receptor clones are depicted in Figure 14 part 'B'. The most 3' 789 bases of the 3.6 Kb (Xho I) clone were identical to the 5'-portion of the 2.1 Kb (Acc I-Sac I) fragment of the dopamine D5 gene. The UTR sequence immediately 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 -1919 [Marchese et al., 1995]. Approximately, 2300 bp 5' of the translational start site was a small dinucleotide repeat, termed 'TC'13. Also of
Figure 13. Southern blot analysis of human genomic clones of the D5 5'-flanking region. The top panel shows genomic fragments excised out of λgt 11 with Xho I. The 5.0 kb fragment corresponded to the D5 coding region. The results of southern blot analysis shown below, reveals a 3.6 kb fragment after hybridization with a ^32P-labelled Acc I-Sac I fragment that encoding the D5 coding and 5'-flanking regions. Size markers (kb) are shown in lane 1. Lanes 2-5 are phage clones digested with Xho I restriction endonuclease
cont'd
Figure 14. **Part A.** Restriction map of the 5'-flanking region of the human D5 receptor gene. Unique restriction endonuclease sites and those used for subcloning purposes are depicted. **Part B.** Nucleotide sequence of the 5'-flanking region of the D5 receptor gene. The most 5' end of the D5 coding region is designated as +1 and the 5'-flanking regions most 3' end is denoted as -1. Two anti-sense Alu sequences are present between nucleotides -3244 to -2953 and -2624 to -2315, 5' to the translational start site.
note, there existed 2 anti-sense Alu sequences between nucleotides -3244 to -2953 and -2624 to -2315, 5' to the translational start site [Marchese et al., 1995]. Both these Alu sequences showed a high degree of conservation in their direct repeat regions and their 'A'-rich regions contained an extra adenine residue. Figure 15 provides a sequence alignment of these Alu sequences, a human consensus Alu sequence and 7SL RNA.

ii.) PCR of cDNA Libraries - A schematic of the regions of the D5 5'-flanking sequences and the oligonucleotides used in PCR amplifications of human caudate, putamen and temporal cortex cDNA libraries is depicted in Figure 6. Southern blot analysis of PCR reaction products after amplification of a putamen library run on a 0.8% agarose gel revealed that only one oligonucleotide pair (P13 and P15, reaction #4) efficiently amplified a D5 5'-flanking cDNA clone (Figure 16). This positively hybridizing band was identified using the Xho I 3.6 kb genomic fragment Nick-translated with 32P-dCTP (Figure 16, lane 5). This amplification product was also seen after amplification of the temporal cortex library using Southern blot analysis but was not visible using ethidium bromide staining techniques (data not shown). The corresponding band from the putamen reaction was excised from a low-melt agarose gel, blunt-ended, ligated into the vector pSP73 and sequenced in both directions.

The oligonucleotide pair used to generate this clone was targeted to 5'-flanking genomic sequences encompassing the region between -1354 and -895 bp, relative to the translational start site. Sequence analysis of the cDNA clone obtained revealed that the amplified clone was 460 bp (termed 'putamen-460') in length and had 100% homology with bases -1354 to -895 of the genomic 5'-flanking sequence of the D5 receptor. No other oligonucleotide pair was able to sufficiently amplify D5 5'-UTR cDNAs. One other extremely faint hybridizing band was observed after Southern blot analysis (note lane 4 to the immediate left of the lane with the strongly hybridizing band in Figure 16), but repeated
Figure 15. **Comparison of Alu sequences and 7SL RNA.** The Alu consensus sequence (Alu Cons) was taken from Jurka and Smith [1988] and is a composite consensus sequence determined from 125 human Alu sequences. Alu-A was found in the D5 5'-flanking region between nucleotides -3244 and -2953, and Alu-B was found between nucleotides -2624 and -2315.
Figure 16. Southern blot analysis of PCR products amplified from human putamen cDNA libraries. Above shows cDNA clones obtained by PCR using primer pairs illustrated in Figure 6. After Southern blot analysis (lower panel) an approximately 460 bp band can be visualized after hybridization to the Xho I 3.6 kb probe in lane 5. Size markers (kb) are shown in lane 1. Lanes 2-7 correspond to reactions 1-6 (see Figure 6).
 attempts at to re-amplify enough of this product for subcloning and sequence analysis were unsuccessful. Furthermore, this hybridizing band was so weak (it was readily visible after EtBr-staining) that it probably did not represent specific binding of the probe to a D5 cDNA clone.

iii.) cDNA Library Screening with D5 Receptor Gene 5'-Flanking Sequences - 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. Human caudate, putamen and temporal cortex libraries were screened with Nick-translated 32P-dCTP-labeled 'putamen 460' bp fragment and later, the Xho I 3.6 kb genomic fragment. All screens using the 'putamen 450' bp fragment yielded only weakly hybridizing clones. Several positively hybridizing clones from each screen were picked, isolated, subcloned into pSP73 and sequenced. Sequence analysis of all these clones demonstrated that none corresponded to the D5 5'-flanking sequence. Clones obtained by homology screening with the Xho I 3.6 kb genomic fragment yielded several strongly hybridizing positive clones. However, after isolation and sequencing none of the clones obtained contained any regions with high sequence identity to the D5 5'-flanking genomic sequence.

iv.) Cloning by 5'-RACE - 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 17). After performing the 5'-RACE reaction (using oligonucleotide P3 as a D5-specific primer), ethidium bromide-stained agarose gel electrophoresis and Southern blot analysis revealed an approximately 400 bp sized band (see Figure 18). This cDNA was subcloned into pSP-73 and sequenced. After following the manufacturer's protocol, the most 5' end of a cDNA clone
should be preceded by a poly-cytosine tail (see Figure 7). The sequence information obtained from this clone indicated that the cDNAs most 5'-terminus corresponded to base -2125 of the genomic sequence. An autoradiogram of one of the sequencing reactions of this clone in the region of its poly-cytosine tail is presented in Figure 19. 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. A second hybridizing band can be visualized in Figure 18. This section of the agarose gel was excised and re-amplified with the universal amplification primer and primere P3. Clones obtained after PCR re-amplification were also subcloned and sequenced. Sequence analysis demonstrated that it had a high degree of homology to the D5 5' genomic sequence with the exception that its most 5' end had a high degree of homology with primer P3. Examination of the D5 5'-flanking sequence in that region contained some similar sequences to that of primer P3 and therefore, the clone obtained was considered to represent a PCR artefact. Several clones were sequenced and all were identical. Figure 22 provides a nucleotide sequence map of the D5 receptor gene that illustrates the transcriptional start site.

In an attempt to obtain cDNA clones from the entire 5'-UTR, cDNA's from the first-strand synthesis reaction were subjected to PCR amplification with D5-specific oligonucleotides within this region. Using oligonucleotides P4 and P5 (see Figure 17) a 978 bp cDNA was identified in EtBr-stained agarose gel-electrophoresis and Southern blot analysis, as illustrated in Figure 20. The cDNA clone obtained was identical to the D5 genomic sequence from nucleotides -1853 to -876. Attempts to obtain cDNA clones corresponding to the area between nucleotide -876 of the genomic sequence and the coding region using a 5'-RACE assay system were unsuccessful.
Figure 17. Schematic of the D5 receptor 5'-flanking region. Restriction endonuclease sites are denoted: X (Xho I); E (Eco RI); A (Acc 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).
Figure 18. Southern blot analysis of 5'RACE products. The left panel shows bands of PCR products amplified with primers P3 and UAP (see Figure 17) after first-strand synthesis with the D5-specific primer D5-SP1. In the right panel are shown bands that hybridized to the $^{32}$P-labelled oligonucleotide directed to sequences immediately 5' to the primer P3. Size markers (kb) are shown to the left of each lane.
Figure 19. Sequence autoradiogram depicting transcriptional initiation site of the D5 receptor. Sequencing of the complementary strand of the 381 bp RACE-generated cDNA on the right and sequence of human genomic DNA on the left reveals the site for transcription initiation corresponds to the thymidine residue at position -2125bp.
Figure 20. Southern blot analysis of nested 5'-RACE products. The left lane shows bands of PCR products amplified with primers P4 and P5 (see Figure 17) after first-strand synthesis with the D5-specific primer D5-SP1 and Superscript™ reverse transcriptase. The right panel is shown the approximately 960 bp band that hybridized to the 32P-labelled oligonucleotide directed to sequences immediately 5' to the primer P5. Size markers (kb) are shown to the right of each lane.
v.) RT-PCR of Human Post-mortem Total RNA - Several attempts were made to obtain cDNA clones encompassing the region between nucleotide -876 and the coding region 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 artefactual. Southern blot analysis demonstrated that any given PCR amplification yielded a population of clones of several different sizes (data not shown). However, even clones of apparently the same size displayed different splicing patterns. In fact, few clones so-called intron/exon 'splice junctions' had correct splice consensus sequences according to Mount [1982]. As stated previously, 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.

vi.) cDNA Cloning by Expression in COS-7 Cells - In order to obtain cDNA clones between nucleotide -876 and the coding region, a 5.7 kb (Eco RI) genomic fragment containing the entire coding region and 5'-UTR of the D5 receptor gene was inserted 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 and primer P7 (page 41). Using either oligonucleotides P6 and P10 or P8 and P9, and 7-deaza-dGTP (Fig 17) 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 and ethidium bromide staining. Figure 21 shows the PCR products amplified by primer P6 and P10 after electrophoresis and ethidium bromide staining. The cDNA band visible in lane 1 of the bottom panel was subcloned into pSP73 and sequenced entirely in both directions. The primers (P6 and P10) used in reaction #5 (see Figure 8) were designed to amplify the
Figure 21. Agarose gels electrophoresis of PCR reaction products from COS-7 cell expression studies. Lane 3 in the top panel shows products amplified between the region -901 and +26 bp using primers P6 and P10 (see Figure 17) from a genomic clone template. Lane 1 of the bottom panel shows the clones obtained using the same primers but from reverse transcribed total RNA from COS-7 cells transfected with the Eco RI 5.7 kb-pCD construct. All amplification reactions were carried out using 7-deaza-dGTP.
region between nucleotides -924 and +26 in the D5 5'-flanking and coding regions. These primers satisfactorily amplified this region from a genomic template (see lane 3 in Figure 21 in the gel labeled 'Genomic DNA'). The cDNA band in the left lane of the lower gel depicted in Figure 21 corresponds to the cDNA obtained after RT-PCR of total RNA derived from transfected COS-7 cells using the same set of primers. This cDNA appears to be approximately 100-200 bp smaller than the genomic clone.

At least 6 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. A nucleotide sequence map is shown in Figure 22, and displays the positions of the intron/exon boundaries identified via cDNA cloning of COS-7 expressed D5 RNAs. These data indicated that the D5 receptor gene 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'-terminus of the coding region.
cont'd.
Figure 22. Nucleotide sequence of the 5'-UTR and 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 bold script-italics indicate intron/exon acceptor/donor sites. Lower case letters in plain text denote promoter and 5'-flanking non-coding sequences. Sequences studied for promoter activity are underlined. 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.
2. **Distribution of D5 Receptor mRNA**

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. A nucleotide sequence alignment of this region of the D5 and the D5 pseudogene 5'-flanking regions is given in Figure 23a. 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 3 provides a summary of the D5 receptor mRNA distribution and relative message abundance in human and old world monkey brain slices.

i.) **Northern Blot Analysis** - In order to determine if the region between nucleotides -2125 to -1920 of the D5 5'-UTR could be used as a more specific D5 probe for *in situ* hybridization studies, cRNA probe corresponding to this region were used in Northern blot analysis and results from this work were compared to those using a probe directed to the D5 gene coding region in Northern blot analysis. The results of Northern blot analysis using [³²P]-end labelled probes directed either to the D5 coding region or the 5'-end of the D5 UTR are presented in Figure 23b. Analysis using *Sma I-Sac I* restriction endonuclease probe (the coding region probe) detected 4 distinct message sizes after Northern blot analysis. These transcripts ranged from approximately 3.0 to 8.0 kb in length. The sizes of the messenger RNAs observed is in agreement with the observations made by Laurier et al. (1994). After Northern blot analysis with the 198 bp 5'-UTR probe (the 'D5-specific' probe), only two of these transcripts were detected. One of these transcripts was approximately 4.0 kb in length and the other approximately 3.0 kb in length. Whether both these transcripts correspond to D5 mRNA was not ascertained.
### TABLE 3. Dopamine D5 Receptor mRNA Distribution in Old World Monkey and Human Brain Slices

<table>
<thead>
<tr>
<th>Region</th>
<th>Monkey</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Ganglia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudate</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Putamen</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Claustrum</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Globus Pallidus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hippocampus and Related Structures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dendate Gyrus</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Hippocampal Subfields</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Subiculum</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Entorhinal Cortex</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Amygdala</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Thalamus (anterior)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pulvinar</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Lateral Geniculate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Neocortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>superficial layers</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>deep layers</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Sections of human and old world monkey brain were incubated with a 5' [35S] labelled 198 bp riboprobe that shared no sequence identity with either of its two pseudogenes. Areas denoted with '++++' expressed the highest levels of D5 mRNA, areas denoted by '++' contained moderate levels, '+' denote sparse labeling and areas denoted with '-' contained no appreciable message. Slices from 4 different human brains and 3 different monkey brains were used for in situ analysis, each of which provided similar results.
**ii.) In Situ Hybridization in Old World Monkey Brain Slices** - Figure 24 shows D5 receptor mRNA distribution in old world monkey brain. Results obtained from 3 different brains were similar with respect to the distribution and labelling intensity. Coronal sections of old world 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 in 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 in the periaqueductal gray (CG).

**iii.) In Situ Hybridization in Human Brain Slices** - Figure 25 depicts D5 receptor mRNA distribution in human brain. Results obtained from 4 different brains were consistent with respect to the distribution and intensity of the signals. 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 26 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 labelling 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), entorhinal cortex (erc) and the neocortex (neo). Part 'B' demonstrates in more detail the labelling seen in neocortex, with highest D5 receptor mRNAs levels seen in layers II, IV and VI.
Figure 26. Detailed micrographs of \textit{In situ} hybridization of D5-receptor mRNA in human brain. Human brain sections were incubated with a 5' [\textsuperscript{35}S]-labelled 194bp riboprobe under condition described in Materials and Methods. Panel A is at the level of the hippocampus and reveals intense labelling 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 can be seen in CA1 (1), the subiculum (S), entorhinal cortex (erc) and the neocortex (neo). Panel B - provides more detail of the labelling seen in neocortex, with highest D5 receptor mRNAs levels seen in layers II, IV and VI.
3. Functional Analysis of the D5 Gene Promoter Region

i.) DNA Structure of the D5 Promoter Region - The region immediately 5' to the transcription initiation site did not contain 'CAAT' or 'TATA' motifs (see Figure 22). The D5 promoter region is not 'GC'-rich and appears to have one discrete transcriptional start site. This region of the D5 gene also contained multiple transcription factor-binding consensus sequences. Between nucleotides -2532 and -2523 existed an identical consensus sequence to the Spl site in the Harvey ras 1 proto-oncogene promoter [Ishii et al., 1986], termed Sp1-a, and a perfect Sp1 consensus sequence [Briggs et al., 1986] between nucleotides -2467 and -2462, termed Sp1-b. Also, an 11 base-pair sequence identical to the pituitary-specific human growth hormone factor, Pit-1/GHF-1 [Guitierrez-Hartmann et al., 1987; Lefevre, et al., 1987; Nelson et al., 1988] existed between nucleotides -2211 and -2201. Furthermore, two sequences similar to the consensus sequence for the transcription factor AP1 [Angel et al., 1987; Lee et al., 1987] were seen between nucleotides -2197 to -2191 and -2185 to -2178, termed AP1-a and AP1-b, respectively. Finally, a degenerate glucocorticoid response element [Tsai et al., 1988; Evans and Arriza, 1989] was observed overlapping the putative transcriptional start site between nucleotides -2131 and -2117. The elements listed above are highlighted in the nucleotide sequence depicted in Figure 22. Table 4 provides the location and nucleotide sequence of these sites as well as the originally characterized consensus sequences.

ii.) RT/PCR of Mammalian Cell Lines - In order to find a suitable cell line to study the transcriptional activation of the D5 receptor gene using the luciferase gene reporter system, several cell lines were subjected to RT-PCR. Table 5 provides a list of the cell lines whose RNA was extracted and subjected to RT-PCR in order to determine if any of the cell lines expressed the D5/D1b gene product. None of the cell lines tested appeared to express D5 (or D1b for the mouse and rat cell lines). Furthermore, radioligand binding
**TABLE 4.** DNA sequence motifs with possible regulatory function in the human D5 receptor gene promoter*

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Consensus</th>
<th>Sequences</th>
<th>Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp1(ras1)</td>
<td>GCTCCGCCTC</td>
<td>GCTCCGCCTC</td>
<td>-2532 to -2523</td>
</tr>
<tr>
<td>Sp1</td>
<td>CCGGCC</td>
<td>CCGGCC</td>
<td>-2467 to -2462</td>
</tr>
<tr>
<td>Pit-1/GHF-1</td>
<td>TGTAAATATT**</td>
<td>TGTAAATATT</td>
<td>-2211 to -2201</td>
</tr>
<tr>
<td>AP-1</td>
<td>TGAATCA</td>
<td>TGAATCA</td>
<td>-2197 to -2191</td>
</tr>
<tr>
<td>GRE</td>
<td>TGTACANNTGTCT</td>
<td>TGACTTGTCTTCT</td>
<td>-2131 to -2117</td>
</tr>
</tbody>
</table>

*references in text
**core sequence in the growth hormone promoter [Lefevre et al., 1987]*
TABLE 5: **RT/PCR of D5/D1B Receptor mRNA and D5 promoter-induced luciferase activity in various mammalian cell lines.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Species</th>
<th>RT/PCR</th>
<th>Luciferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>B 104</td>
<td>Rat</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RINM</td>
<td>Rat</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rat 1</td>
<td>Rat</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC 12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B-TCE</td>
<td>Mouse</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NB41A3</td>
<td>Mouse</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P19EC</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>COS-7</td>
<td>Monkey</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>Hamster</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T671</td>
<td>Human</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IMR32</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GH3</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

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.
assays did not reveal the presence of a D5 receptor population in the SK-N-SH and COS-7 cell lines (data not shown). In light of this information, several cell lines were transiently transfected with the chimeric D5-luciferase plasmid constructs in the hope of finding a 'permissive' cell line.

iii.) 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 5'-UTR of the D5 gene could drive transcription in the presence of the necessary nuclear factors. The results of the luciferase assays using this 5'-UTR and also deletion mutants of the 5'-UTR of D5 in SK-N-SH and GH3 cells are presented in Figure 27. 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 (3.5X in SK-N-SH cells; 25X in GH3 cells) 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) between bases -2626 and -2387, the nucleotide sequence in p500D5-LUC that was deleted in the p251D5-LUC construct (see Fig 27A). 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 2X or 0.5X of that seen in the negative control in GH3 and SK-N-SK cells, respectively. These activities were about 15% of the maximal activity seen with the p251D5-LUC construct.

D5 promoter activity displayed a cell-specific pattern of expression. The results of our luciferase assays using different mammalian cell lines are presented in Table 5. Seven
different mammalian cell lines were tested for luciferase activity using the 5 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 (data not shown).

iv.) Effect of Forskolin and Phorbol Esters - Consensus sequences for the TPA and forskolin-inducible transcription factor AP1 were identified between nucleotides -2197 to -2191 and -2185 to -2178. To test if these sequences within the human D5 receptor gene promoter bound AP1, the human neuroblastoma cell line SK-N-SH was transfected with the D5 promoter-luciferase chimeras p500D5-LUC, or p251D5-LUC. Following transfection cells were treated with either 0.1 μM TPA, 1.0 μM TPA, 5.0 μM forskolin or 0.1 μM TPA and 5.0 μM forskolin. In all instances, TPA and forskolin either alone or in combination failed to elicit a significant change in D5 promoter-induced luciferase activity, as compared to vehicle-treated transfectants (see Figure 28). Cells were also treated with TPA and forskolin before transfection, and again no change in luciferase activity was observed. The pituitary cell line was also treated with TPA following transfection with D5-luciferase chimera plasmids and again no noticeable change in luciferase activity as compared to vehicle-treated cells was observed (data not shown). Therefore, it seems that even though several highly conserved consensus sequences for the transcription factor AP1 exist within the sequences of the D5 receptor's proximal promoter region, AP1 itself may be unable to bind to either of these sites.
Figure 28. Relative luciferase activity of the p500 and p251 D5-luciferase constructs in human SK-N-SH cells after treatment with TPA and forskolin. 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 % increase above control values. Error bars represent the standard error of the mean. All values obtained were normalised to the β-galactosidase activity of the extract.
v.) *Effect of Dexamethasone* - In order to determine if glucocorticoids affect D5 transcription via binding of the glucocorticoid receptor to a degenerate glucocorticoid response element (GRE) overlapping the putative transcriptional start site, the glucocorticoid receptor-expressing cell line ROS 17/2.8 were transfected with p51D5-LUC followed by addition of either 100 nM, 500 nM, or 1 μM dexamethasone. The results of these experiments are presented in Figure 29. Again, extracts from cells treated with dexamethasone did not demonstrate significantly different D5 promoter-mediated luciferase activity from that of extracts from cells treated with vehicle. These data tend to indicate that the D5 receptor gene expression is not under the influence of the glucocorticoid receptor activity. Figure 30 provides a schematic summary of the D5 promoter deletion mutants tested in the induction studies described above and lists a summary of the results.

vi.) **Electrophoretic Mobility Shift Assays** - In order to determine if nuclear proteins interact with DNA elements within the proximal promoter region of the D5 receptor gene, electrophoretic mobility shift assays were performed using oligonucleotides homologous to various regions of the promoter and nuclear extracts from SK-N-SH and GH3 cells. Double-stranded oligonucleotide GS-1 was designed to be homologous to the region between bases -2562 to -2499 and to encompass the sequence termed Sp1-a, while oligonucleotide GS-2 was complementary to base -2498 to -2431 and included the sequence termed Sp1-b. GS-3 included the 'TC' dinucleotide repeat region and spanned from -2293 to -2250. Finally, oligonucleotide GS-4 was complementary to bases -2245 to -2177 and included the putative Pit-1, AP1-a and AP1-b sites.
Figure 29. Relative luciferase activity of the p51 D5-luciferase construct in human ROS 17/2.8 cells after treatment with dexamethasone. 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 % increase above control values. Error bars represent the standard error of the mean. All values obtained were normalised to the β-galactosidase activity of the extract.
Effect of Hormones and AP-1 Activators on D5-Mediated Luciferase Activity

Figure 30. Summary of results of D5 promoter induction studies. The above schematic illustrates the D5 promoter deletion mutants used in induction studies and the relative position of the putative cis-acting elements identified. Results of each study are listed on the right (not all data shown). In each case no induction of D5 promoter-mediated luciferase activity was observed.
a.) The D5 Negative Modulator Region - A representative autoradiogram of the EMSA performed to investigate possible binding to the Sp1-a site is presented in Figure 31. Using GS-1 as a probe, two retarded bands were observed in the absence of competitors, using nuclear extract from SK-N-SH cells. Both these bands seemed to represent specific binding since they were not competed off by increasing poly-dI.dC concentrations but did disappear in the presence of increasing amounts of cold competitor probe. However, in competition assays using oligonucleotides harbouring various transcription factor consensus sequences listed in Table 2, the upper band completely disappeared in every case and resolution of the lower band diminished (data not shown). This may be the result of weak binding affinity of the nuclear protein for the cis-acting element, or that the observed bands do not really represent specific-binding. It is therefore uncertain, whether Sp1 or a related protein is binding to the putative Sp1-a element within this site. HeLa nuclear extract revealed a similar pattern of retarded bands as was seen after incubation with SK-N-SH extract. HeLa extract is relatively abundant in Sp1 protein and would be expected to give a rather large signal after incubation with probe that contains a perfect Sp1 consensus sequence. This was not the case, suggesting that the putative Sp1 site within this region does not interact with Sp1.

Gel-shift analysis using the GS-2 oligonucleotide probe revealed one specific band as determined by competition with 100-fold molar excess of cold probe (see Figure 32). However this band (band 'S'), was also competed off by cold GS-1 but not cold GS-3, or any of the transcriptions factor consensus sequence oligonucleotides. This suggests that this binding site either overlaps the boundary between GS-1 and GS-2, or that the protein binding to GS-2 also interacts with a similar site in GS-1. The latter is unlikely, since the only motifs the two share are the respective Sp1 sites which do not seem to bind protein at either site as determined by competition with cold Sp1 consensus sequences. If the site overlaps the oligonucleotides boundaries, then this would give credence to the suggestion
Figure 31. **Binding of proteins to the GS-1 ds-oligonucleotide probe.**
Nuclear extracts from SK-N-SH cells were incubated with labeled GS-1 in varying concentrations of dI.dC and cold competitor oligonucleotide. The complexes formed were analyzed in a gel retardation assay. Despite the presence of 8 μg of poly dI.dC, two complexes (S) presumed to represent specific interactions were observed. The formation of both complexes appeared to be significantly inhibited in the presence of 100-fold molar excess of cold competitor GS-1 oligonucleotides.
Figure 32. Binding of proteins to the GS-2 ds-oligonucleotide probe. Nuclear extracts from SK-N-SH cells were incubated with labeled GS-2 with different transcription factor consensus sequence competitor oligonucleotides. The complexes formed were analyzed in a gel retardation assay. The formation of the only specific complex (S) observed appeared to be significantly inhibited in the presence of 100-fold molar excess of cold competitor GS-2 and GS-1 oligonucleotides but not GS-3. Competitor oligonucleotides for common transcription factor were unable to affect the DNA-protein complex formation.
that the GS-1 retarded band that disappears after competition with several cold competitors is binding protein only weakly, as much of the sequence information it requires is missing.

Competition reactions using Sp1 consensus oligonucleotides failed to inhibit the binding of nuclear protein to GS-2, suggesting again that Sp1 does not interact with the D5 promoter in this region. The binding pattern seen after incubation with SK-N-SH extract is identical to that seen with HeLa cell nuclear extract.

b.) The D5 transactivation domains - Deletion mutation analysis using a luciferase gene reporter system localized the D5 transactivation domains to at least two regions (see Figure 27B; Table 6). The first lies between nucleotides -2307 to -2246 and the second between bases -2245 to -2177. In order to investigate the nuclear protein-DNA interactions in these regions, probes GS-3 (with sequence information to the most distal region) and GS-4 (identical to the proximal region) were synthesized and incubated with SK-N-SH and GH3 cell nuclear extract. Two retarded bands were observed after electrophoresis with the GS-3 probe in the absence of competitor. Additions of 50- and then 100-fold molar excess reveals that only the lower band represents a specific DNA-protein interaction (see Figure 33). No putative cis-acting consensus sequences were observed in this area and the commercially available competitor oligonucleotides were unable to compete for the site on the GS-3 probe being bound by the nuclear protein. An autoradiogram from one of these assays is shown in Figure 34. The EMSA pattern observed with SK-N-SH extract was identical to those seen with GH3 and HeLa cell nuclear extracts, as shown in Figure 35.

The transactivation domain identified corresponding to the GS-4 probe was active only in transfection experiments using GH3 pituitary cells (see Figure 27). Within this region there is a consensus site for the pituitary-specific transcription factor Pit-1. Just 3' to this site was a putative AP1 consensus sequence. After incubation of [32P]-GS-4 with
Figure 33. Binding of proteins to the GS-3 ds-oligonucleotide probe. Nuclear extracts from SK-N-SH cells were incubated with labeled GS-3 in varying concentrations of dI.dC and cold competitor oligonucleotide. The complexes formed were analyzed in a gel retardation assay and two complexes were observed (NS and S). The formation of the lower complex (S) appeared to be significantly inhibited in the presence of 100-fold molar excess of cold competitor GS-3 oligonucleotide, while the upper complex (NS) was not, suggesting a non-specific interaction.
Figure 34. Gel shift competition studies using the GS-3 ds-oligonucleotide probe. Nuclear extracts from SK-N-SH cells were incubated with labeled GS-3 with different transcription factor consensus sequence competitor oligonucleotides. The complexes formed were analyzed in a gel retardation assay. The formation of the specific complex (S) observed appeared to be significantly inhibited in the presence of 100-fold molar excess of cold competitor GS-3. Competitor oligonucleotides for common transcription factor were unable to affect the DNA-protein complex formation. HeLa cell nuclear extract (far righthand lane) produced an identical banding pattern as that observed after incubation with SK-N-SH nuclear extract.
Figure 35. Comparison of GS-3 oligonucleotide-protein complexes with SK-N-SH and GH3 nuclear extracts. SK-N-SH and GH3 nuclear extracts were incubated with labeled GS-3 ds-oligonucleotide probe and complexes were analysed in a gel retardation assay. Using an equal amount of nuclear extract the retarded complex banding patterns appeared to be identical.
GH3 nuclear extract 3 retarded complexes ('A'-'C') were easily observable (see Figure 36) and a much slower migrating complex ('D') is only visible to the naked eye and could not be reproduced photographically. The 3 readily visible complexes were also seen after incubation with SK-N-SH extracts but not the higher fainter complex. All four complexes seemed to be competed off by 100-fold molar excess of cold competitor oligonucleotide. The Pit-1 consensus oligonucleotide appeared to compete for a nuclear factor binding to GS-4 and seen as the retarded complex 'B'. Furthermore, AP1 consensus oligonucleotide appeared to interfere with the DNA-protein interaction represented by the retarded complex 'C'. The relative contribution these complexes make to the transactivation of the D5 receptor gene is unknown as SK-N-SH nuclear extract reveals a nearly identical mobility shift pattern, and this region acts only as a relatively weak transactivator in the SK-N-SH cell line.
Figure 36. Binding of proteins to the GS-4 ds-oligonucleotide probe. Varying amounts of nuclear extracts from GH3 and SK-N-SH cells were incubated with labeled GS-4, with or without either 100-fold molar excess of unlabeled GS-4, Pit-1 or AP1 consensus sequence competitor oligonucleotides. The complexes formed were analyzed in a gel retardation assay. The formation of three major specific complexes (A, B, and C) and other less resolved complexes observed appeared to be significantly inhibited in the presence of 100-fold molar excess of cold competitor GS-4. Complex C appeared to be significantly inhibited by AP1 consensus oligonucleotides (competitor A-bottom row). Formation of complexes A and B and other complexes between them appeared to be inhibited in the presence of Pit-1 consensus oligonucleotides (P). Unappreciable was a high molecular weight complex observed after incubation with GH3 nuclear extract but not SK-N-SH.
vii.) **SSCP analysis and cloning of human genomic DNA's** - Thirty-six human chromosomes from 7 schizophrenic, 6 Huntington's, and 2 Parkinson's patients and 3 healthy controls were examined to determine if the 'TC' dinucleotide repeat region in the D5 gene promoter region was polymorphic. Genomic DNA was amplified using PCR and 2 D5 gene-specific oligonucleotide primers (termed P1 and P2). These primers amplified a portion of the D5 gene promoter between bases -251 and -97 upstream of the transcription initiation site (see Figure 12), yielding a 154 bp clone when amplifying the wild-type D5 promoter. These PCR products were electrophoresed in a 5% non-denaturing polyacrylamide gel to perform single-strand conformation polymorphism analysis. SSCP analysis revealed 3 distinct banding patterns. Figure 37 shows a representative silver-stained acrylamide gel of the electrophoresed SSCP reaction products. Fifteen of the individuals examined displayed an identical pattern to that seen with PCR products from amplification reactions of the similiar region from the D5 5'-flanking genomic clone obtained from a lambda EMBL SP6/T7 library [Beishlag et al., 1995]. Lane 2 of figure 37 displayed an extra band after SSCP analysis. One individual was schizophrenic and the other with an identical banding pattern was a Huntington's disease patient (data not shown). Less easy to appreciate is the banding pattern in lane 4 of figure 37. This individual (also schizophrenic) repeatedly displayed a variant banding pattern after SSCP analysis readily definable by the naked eye.

The genomic DNAs from the individuals who displayed variant banding patterns were amplified again using oligonucleotide primers P1 and P3. These primers amplified a region within the genomic sequence 251 bp 5' of the transcription initiation site and 201 bp 3' of this site (see Fig 12), yielding a 452 bp clone from wild-type samples. The products of the PCR reactions were subcloned and sequenced. Sequence analysis revealed that the schizophrenic and Huntington's patients with identical banding patterns seen in the SSCP analysis contained only 12 'TC' repeats in their genomic sequence in at least one
Figure 37. Products of the PCR for the common and variant DNA's of the D5 promoter region. Shown above are the silver-stained PCR products of the D5 repeat region from 4 schizophrenic patients genomic DNAs after SSCP analysis. The vertical arrow indicates the direction of migration of the PCR products along the electrical field. One schizophrenic patient's conformation pattern revealed an easily identifiable extra band (Arrow 'A' - lane 2). One Huntington's patient had an identical pattern to that of subject #7 (data not shown). More difficult to appreciate is the variant banding pattern of a schizophrenic patient in lane 4. One band seen in all other lanes is missing (Arrow 'B') and the top band appears to be a doublet (Arrow 'C').
Figure 38. Cloning and sequence analysis of the D5 promoter polymorphic repeat region - Sequence autoradiogram of the common and variant clones of the dinucleotide repeat region of the human D5 receptor gene promoter. A segment of the D5-5'-flanking region, encompassing 251 bases upstream (5') of the transcription initiation site, and 201 bases downstream (3') of this position was generated by PCR of genomic DNAs, using oligonucleotide primers P1 and another primer, P3 (see Figure 12). This fragment was then blunt-ended and subcloned into the luciferase gene-containing plasmid PL(KS)b-LUCnPL to create the D5 luciferase mutants TC12LUC and TC14LUC. Clones were sequenced in both directions by the dideoxy chain-termination method described by Sanger et al., [1977] using a Sequenase version 2.0, 7-deaza dGTP sequencing kit (United States Biochemical, Cleveland, Ohio).
chromosome. The other schizophrenic displaying a variant SSCP banding pattern contained 14 'TC' repeats within the genomic sequence in at least one chromosome (see Figure 38).

viii.) Transient transfection of D5-luciferase constructs - The clones obtained from PCR of the variant subjects human DNAs were fused in front of the firefly luciferase gene contained in the plasmid vector PL(KS)b-LUCnPL. These plasmids were tested using a transient transfection method to determine luciferase activity. The values obtained for the (TC)_{12}, (TC)_{14} and the wild-type-LUC chimeras are presented in Figure 39. The luciferase activities for the allelic D5 promoter-LUC chimeras were not significantly different from the activity seen in the wild-type. This suggests that, in our assay the insertion or deletion of one 'TC' does not have any functional consequence for transactivation.
Figure 39. **D5 promoter mutant allele luciferase assays** - Relative luciferase activity of the common and variant forms of the human D5 receptor gene promoter/luciferase chimeras in the human neuroblastoma cell line SK-N-SH. These values represent the means of three independent experiments performed in duplicate. Error bars represent the standard error of the mean (+/-s.e.m.)
V. Discussion

This work describes for the first time the genomic organization of the 5'-flanking region and promoter structure of the human dopamine D5 receptor gene. Furthermore, it was demonstrated that the region immediately 5' to the putative transcription start site can mediate transcription in a heterologous reporter gene system. The major enhancer and silencer regions in the proximal promoter have been identified, as has a polymorphic dinucleotide repeat. Also presented in this thesis is the first in situ hybridization data of the human D5 receptor mRNA using 5' probes distinct in nucleotide sequence from the two D5 pseudogenes. As such, this work provides the first unequivocal data on the region-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 the nuclear factors involved in its transcriptional regulation may aid in our understanding of the role this gene product plays in the generation and maintenance of the dopamine D1-like receptor mediated events.

1. Cloning of the D5 5'-Flanking Region

i.) Genomic DNA 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. In its coding region, the D5 gene predicts an amino acid structure that maintains 83% identity with that of the rat D1b receptor, the 'D5 orthologue' in rodents (Tiberi et al., 1991). This degree of identity dropped off significantly in the 5'-flanking region. Tiberi and his colleagues also reported sequence information for the D1b gene extending 693 bp 5' of the start of coding; an alignment of this sequence and the D5 5'-flanking sequence is presented in Figure 40. The D5 5' gene sequence had 57% identity to the 5'-flanking sequence of the rat D1b gene. This apparent divergence in sequence identity could potentially mean that the human D5 5'
**Figure 40.** DNA nucleotide sequence alignment of the human D5 and rat D1b 5'-flanking regions. The 5'-flanking sequence (693 nt) reported by Tiberi et al. (1991) aligned with corresponding region of D5 reveals 57% identity between the two regions. Identities are boxed and gaps have been introduced to maximize the alignment.
regulatory elements are not shared with lower species, thereby making non-primates unattractive models for the study of the D5 5'-flanking region and promoter function.

The sequence immediately 5' to the translational start site (-1 to approximately -900) 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 -1919 (see Figure 23) [Marchese et al., 1995]. Also of note, there existed 2 anti-sense Alu sequences between nucleotides -3244 to -2953 and -2624 to -2315, 5' to the translational start site [Marchese et al., 1995].

a.) Alu Sequences - It has been proposed that the human genome contains as many as 700 000 Alu sequences comprising approximately 6% of our genome, many of which are transcribed by RNA Pol III [Reinhart et al., 1981; Hwu et al., 1986; Moyzis et al., 1989]. Alu sequences are easily recognizable because of their high sequence identity with 7SL RNA, a cytoplasmic RNA [Haynes and Jelinek, 1981]. The two sequences present in the D5 5'-flanking region do not appear to be human-specific by nature of their lack of specific point mutations [Batzer and Deininger, 1991; Batzer et al., 1991], suggesting that they pre-date homo sapiens. Another such sequence was found in the 3'-flanking region of the D5 receptor gene, however, its orientation and exact location were not ascertained (data not shown). The presence of Alu sequences in the 5'-flanking region is provocative because of the important role they play in genetic recombination and genetic disease. Alu sequences can do this in several ways; (i) they can provide sites of homologous and heterologous recombination that produce deletions, insertions and chromosomal rearrangements [Lehrman et al., 1985; Myerowitz and Hogikyan, 1987; Nicholls et al., 1987; Markert et al., 1988; Rouyer et al., 1987], (ii) they may undergo de novo retrotransposition into the sequences of other genes [Vidaud et al., 1989], and (iii)
upstream 'antisense' Alu fragments may inserted into the splice junctions of the RNA transcripts of other genes [Mitchell et al., 1991].

b.) **D5 Pseudogenes** - Previously, it had been reported that the human D5 pseudogenes, ψD5-1 and ψD5-2 differed in nucleotide sequence by approximately 5.7% and 6.4% respectively, with the functional D5 gene [Nguyen et al., 1991]. This degree of identity (94%) is maintained in both the 3' and 5'-flanking regions [Marchese et al., 1995]. It has been proposed that once pseudogenes are first silenced, they are under no selective pressure to maintain their nucleotide sequence and hence, mutate at a rate of 1-2 x 10^-9 nt substitutions/site/year [Bailey et al., 1991]. This then would predict that the first pseudogene arose approximately 20 million years ago, while the second event, giving rise to ψD5-2, has been placed at approximately 7.6 million years ago [Marchese et al., 1995]. Figure 41 provides a schematic representation of the time course of the predicted gene duplication events in the human D5 gene family. This approximation is consistent with data demonstrating that the African green monkey, a primate lineage that diverged from the human ancestral lineage more than 25 million years ago [Bailey et al., 1981], lacks D5-pseudogenes [Nguyen et al., 1991]. Moreover, the gorilla genome contains both pseudogenes, and the gorilla has been considered to have shared a common ancestor with man less than 7 million years ago [Marchese et al., 1995]. Furthermore, the presence of the two pseudogenes in the gorilla genome would suggest that the human population is not polymorphic for the presence of the two pseudogenes as was earlier postulated [Nguyen et al., 1991].

Comparison of the D5 gene 3' and 5'-flanking sequences with those of its two pseudogenes reveals that while the pseudogenes maintain a high degree of sequence homology with each other for several kb 5' of their respective coding regions, they lose identity with the D5 gene sequence 5' of nucleotide -1919. Immediately 5' of the point of
Figure 41. Schematic representation of the predicted gene duplication events in the D5 gene family. In the first event, estimated to have occurred 20 million years ago (MYR), D5 duplicated giving rise to the first pseudogene. Subsequently in a second event, estimated to have occurred 7 Myr ago, the pseudogene duplicated giving rise to the second pseudogene.
divergence in the two pseudogene sequences is the 5' portion of an Alu fragment [Marchese et al., 1995]. The presence of this monomeric Alu fragment suggests that at least one pseudogene arose by an Alu-mediated heterologous recombination event similar to the mechanism described above. Sequence identity is maintained between the D5 gene and its pseudogenes 3' of the coding region for at least 14 kb and the 3' point of divergence between the two pseudogenes and the D5 gene has not been identified [Marchese et al., 1995]. It seems quite reasonable that a monomeric Alu fragment (the 3' end corresponding to the 5' portion identified in 5'-flanking sequences of the two pseudogenes) resides at the 3' point of divergence between D5 and the D5-pseudogenes.

ii.) cDNA Cloning - The isolation of cDNA clones corresponding to the D5 5'-flanking region was necessary in order to identify the transcriptional start site(s) and gene structure of the D5 gene. Conventional techniques used to obtain such clones and identify the transcriptional start site(s) include, high stringency homology screening with genomic clones and S1 nuclease assays using DNase I. Complementary DNA libraries available from commercial suppliers such as Clontech and Stratagene are both oligo-dT and random primed, yielding average inserts with an average length of 0.9-1.5 kb. Random priming is far less effective than oligo-dT priming for amplification of RNAs transcribed by RNA polymerase II. This results in the presence of a large number of inserts containing sequence information of the most 3'-ends of most transcripts and may account for the lack of success in obtaining 5'-flanking UTR clones of the D5 gene. Furthermore, as stated in the 'Results' section under 'Human Genomic Library Screening', the Xho I 3.6 kb fragment contained two Alu sequences. Up to 700 000 of these fragments have been postulated to exist in the human genome, many of which are transcriptionally competent [Hwu et al., 1986; Moyzis et al., 1989; Reinhart et al., 1981]. Therefore, cross hybridization within this family of sequences may have accounted for the strength of
hybridization even under high stringency conditions. Moreover, D5 mRNA needed for protection assays was reported to be rare [Mansour and Watson 1995; Meador-Woodruff et al., 1994], and a D5 mRNA-rich tissue source had not been identified. Without the benefit of such a tissue it was unfeasible to 'blindly' consume valuable human post-mortem tissue in an attempt to isolate sufficient D5 mRNA of high quality for use in protection assays. As such, other methods were employed in order to obtain D5 cDNA clones and identify the transcriptional start site of the human D5 receptor gene.

a.) Cloning with 5'-RACE - 5'-RACE was performed in order to determine the 5' transcriptional start site of the D5 receptor gene. Deoxy-cytosine-tailed clones obtained from repeated attempts at 5'-RACE all revealed an identical 5' termini, suggesting that the D5 gene may have only one discrete transcriptional start site. The region immediately 5' to the transcriptional 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]. The area immediately surrounding the transcription initiation site is pyrimidine-rich and may correspond to the initiator region (Inr) described by Smale and Baltimore [1989]. These observations fall in line for 'TATA'-less and 'CAAT'-less promoters that do not have an unusually high 'GC' content, in contrast to 'housekeeping genes' [Mouradian et al., 1994]. This region of the D5 gene also contained several putative transcription factor binding motifs including sites for Sp-1 [Briggs et al., 1986; Ishii et al., 1986], AP1 [Lee et al., 1987], glucocorticoid response element half sites [Tsai et al., 1988], and an 11 base-long sequence identical to the pituitary-specific human growth hormone factor, Pit-1/GHF-1 (Fig 22)[Lefevre et al., 1987; Nelson et al., 1988].
b.) 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 2 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 artefactual. As stated, the area immediately 5' to the D5 coding region was very 'GC' rich and all clones obtained appeared to suggest the presence of "introns" of differing lengths. This was due, potentially, 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. Moreover, the 5'-flanking sequence contained several other 'ATG' codons in frame. However none of these codons corresponded to correct 'Kozak' sequences [Kozak, 1986] (See RESULTS "RT-PCR of Human Post-mortem Total RNA")

In order to obtain cDNA clones between nucleotide -876 and the coding region, the problems of scarce human brain samples, low mRNA expression, high 'GC' content and the existence of transcriptionally competent pseudogenes, were circumvented by inserting a 5.7 kb (Eco RI) genomic fragment containing the entire coding and 5'-UTR of the D5 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, in order to reduce possible anomalies in secondary structure caused by high 'GC' content [McConloque et al., 1988]. Sequence analysis of the cDNA clones obtained that overlapped the region between nucleotide -876 and the coding region demonstrated, for the first time, that the D5
gene has at least 2 exons, and that splice variants for the D5 gene existed. Both of the donor sites and the common acceptor site observed in the D5 cDNA clones are proper splice donor sites according to Mount [1982].

iii. D5 Gene Structure - Based on the observations that the D1 and D5 genes were intronless in their coding regions, it was suggested that both were similar in gene structure to the β2-adrenergic receptor gene [Sunahara et al., 1990; 1991]. However, both the human and hamster β2-adrenergic genes are intronless in their 5'-flanking regions [Kobilka et al., 1987]. A small intron in the 5'-flanking region of the D1 dopamine receptor gene (116 bp) has also been reported [Minowa M., et al., 1992], similar to the observations made of the D5 gene presented in this thesis. These data suggest that the D5 gene structure maintains a high degree of similarity to that of the D1 receptor even outside of the coding region, as opposed to the D2 class of receptors. While the 5'-flanking regions of the D3 and D4 receptor genes have not been characterized, the D2 receptor 5'-flanking region contains an intron in excess of 50 kb (Bunzow et al., 1988). All the genes in the D2-like family contain several introns within their coding sequences, and the D3 and D4 presumably contain more in their 5'-flanking sequences.

Clones of the D5 5'-flanking region suggested that splice variants of the mature D5 mRNA transcript exist. Alternative splicing is considered to be a mechanism for the production of multiple protein isoforms from single genes [see Breitbart et al., 1987], and therefore, have the potential for different selectivities and functions. While splice variants exist for the D2 and D3 receptor [Dal Toso et al., 1989; Fishburn et al., 1993; Giros et al., 1989, 1991], these splice variants give rise to different proteins. The alternative splicing of the D5 message would not alter the putative amino acid sequence of the protein and hence have little effect on function. However, the 5'-UTR may have consequences for D5
mRNA stability, or protein sorting. One mechanism of neuronal polarity (protein accumulation at a given subcellular site) is mRNA targeting. Microtubule-associated protein 2 (MAP2) mRNA accumulates selectively in dendrites and this process is thought to be mediated by cis-acting elements [Garner et al., 1988; Kleiman et al., 1990]. Deletion of part of the exon 1 in the D5 mature mRNA transcript may alter or delete cis-acting elements responsible for mRNA targeting, potentially allowing for the selective targeting of one splice variant to a given subcellular locale. Furthermore, 5'-flanking sequences encoding for peptides (mini-cistrons) are known to inhibit protein synthesis of the β2 adrenergic receptor [Parola and Kobilka, 1994]. As mentioned above, the D5 and β2 adrenergic receptor genes are very similar in gene structure and the D5 gene may be auto-regulated by leader peptides in a similar fashion; any splicing event that would give rise to, or delete such a leader peptide-encoding sequence would be an important mechanism of regulation of the D5 receptor.

The size of the D5 cDNA 5'-UTR is either 1946 bp or 1922 bp (depending on the intron excised). This together with a coding region of 1431 bp predict a message size of at least 3.4 kb (excluding a 3'-UTR and a poly-adenylic acid tract). Sunahara et al. (1991) reported a mRNA species of 3.3 kb while Laurier et al. (1994) observed several bands between 2.3 and 8.0 kb. The smaller message sizes reported do not correspond to the size of the cDNAs reported in this thesis. The different message sizes seen after Northern blot analysis could be the result of alternative transcriptional initiation sites [Kozak, 1988] or corresponds to mRNA encoding for the 2 D5 pseudogenes [Laurier et al., 1994; Nguyen et al., 1991b]. We can be fairly confident that the D5 5'-UTR extends at least to the region between -1.9 and -2.1 kb 5' from the coding region. Using sequence information from this region to construct riboprobes, in situ hybridization analysis revealed a similar pattern of d5 mRNA distribution to that seen using probes directed to the D5 coding region (Meador-Woodruff et al., 1994). Therefore, it would seem that the region between -1.9
and -2.1 kb in the 5'-flanking region encodes D5 cDNA. 3'-RACE was not performed. This type of cloning strategy would allow for the cloning of the most 3' end of the D5 cDNA and with this information a more accurate approximation of the D5 message size could be made.

iv.) Evolution of the D5 Gene - Despite the presence of a 5' intron, the D5 gene structure resembles that of a processed pseudogene. Processed pseudogenes are characterised by colinearity with the coding and UTR regions of existing genes up to, but not beyond the cap signal, a lack of introns, and the presence of an adenylic acid-rich region in the 3'-flanking region [Wagner, 1986]. Sequence analysis of 3'-flanking genomic clones revealed the presence of an adenylic acid-rich region. Figure 42 depicts the nucleotide sequence of the 3'-flanking region starting at the end of coding and reveals the presence of two putative poly-adenylation signal consensus sequences and a poly-A tract. It is via hybridization to these polyadenyl tracts that pseudogenes are thought to arise through a retroviral mechanism [Bernstein et al., 1983; Vanin, 1984]. Once these transcripts are re-incorporated back into the genome they are often silent because they are redundant, or because they lack their native promoter. Most housekeeping genes are transcriptionally competent processed pseudogenes, and this may be a reason why they lack CAAT and TATA boxes [Wagner, 1986]. The lack of CAAT and TATA boxes is a feature they share with the D5 gene. Moreover, this observation is compatible with the fact that deletion mutants of the D5 promoter do not seem to be very powerful transcriptional activators in the heterologous luciferase assays, with the exception of their activity in GH3 cells. Furthermore, the D5 promoter does not seem to be inducible under our experimental conditions in this system.

While there is considerable controversy over whether introns are created or lost during evolution, it is generally accepted that genes arising from retroviral and/or reverse
Figure 42. Nucleotide sequence of the 3'-flanking region of the human D5 receptor gene. Two putative poly-adenylation signal consensus sequences are in uppercase and underlined. An adenylic acid-rich sequence is in uppercase. The adenylic acid-rich sequence could represent the poly-A tail of a mature mRNA that was reverse transcribed through a retroviral mechanism and transposed back into the mammalian genome giving rise to a gene that would become D5.
transcriptional mechanisms lose the introns present in the parent gene. Therefore, the presence of a intron in the 5'-flanking sequence of the D5 gene would argue against such a mechanism being responsible for the creation of D5. However, it also seems possible that introns can be introduced into existing genes. The presence of introns in the mammalian triosephosphate isomerase genes but not in the yeast or bacterial isoforms supports this hypothesis [Marchionni and Gilbert, 1986]. Moreover, the lack of some introns in one of the preproinsulin genes but present in another preproinsulin gene in the rat suggests that some pseudogenes are only partially processed [Soares et al., 1985]. The lack of introns in the many of the G-protein-linked receptor gene families (namely the serotonergic, muscarinic, adrenergic and dopaminergic) is thought to represent a mechanism of evolutionary divergence within a superfamily of genes from a common ancestral precursor gene [O'Dowd, 1993]. Certain structural features of the D5 gene support a role for a retroviral-like mechanism to achieve this diversity. However, the similarity between the D5 and D1 gene structures might suggest that D5 arose from D1 through a DNA recombination event, or vice versa. The presence of a poly-adenylic acid-rich region or any other structural information, in the D1 gene's 3'-flanking region has not been reported. Such evidence would strongly suggest that one of these genes was the direct progenitor of the other, and might provide clues to the mechanism by which these genes arose.
2. *In Situ* Hybridization

The observation that the D5 gene sequence diverged from that of its two pseudogenes 5' of coding, allowed for the development of 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 characterise the distribution of this receptor either by *in situ* hybridization or immunohistochemical techniques. Furthermore, it was demonstrated that at least one pseudogene was transcriptionally competent [Nguyen et al., 1991b; Weinshank et al., 1991]. 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., 1995]. By using this region to make riboprobes, the signals seen in human and old world monkey slices should represent only D5 mRNA.

While the data presented in this work, concerning the distribution of D5 mRNA is consistent for the most part with recently published data [Meador-Woodruff et al., 1994], the lack of identity between the 5'-termini of the D5 cDNA and the pseudogenes allows for better resolution of the D5 signal. There was a relative lack of D5 mRNA in the basal ganglia, a region responsible for motor function and rich in D1 message. Dopaminergic dysfunction in the nigrostriatal system has been identified as the cause of Parkinson's disease (Lee et al., 1978a). Moreover, *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. The apparent presence of D5 message in the pars compacta suggests that the D5 receptor is involved in the nigrostriatal system and potentially the pathology of Parkinson's disease. Lesion studies with 6-OHDA performed
in the substantia nigra failed to reduce D1-like binding sites in the substantia nigra and striatal pathways, and therefore it was suggested that these sites may be presynaptic (Arnt, 1985). This tends to suggest that the sites in question are not nigra-expressed D5 receptors. Furthermore, D1 message is absent in the nigra (Meador-Woodruff et al., 1991; Sunahara, 1993). This lack of correspondence between binding data and in situ hybridization analysis cannot be resolved at present. The D5 protein might be as rare as its message and therefore beyond the detectable range with radio-ligand binding techniques.

Besides the apparent presence of message in the nigra, a similar pattern of distribution was observed in the rat brain for the D1b receptor [Tiberi et al., 1991]. However, there are some other striking differences. D1b seems to very abundant in the rat lateral mammillary nuclei, the parafascicular nucleus, and discrete regions of the hypothalamus [Tiberi et al., 1991], 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 the 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; Levey et al., 1993; Fremeau et al., 1991]. Moreover, 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 receptor subtypes. The absence of the D5 receptor mRNA in the basal ganglia may mean that D5 plays no part in coordinating motor function, unlike D1. The presence of D5 message in the hippocampus and the neocortex supports a role in learning, memory and cognition, [Fremeau et al., 1991; Sawaguchi and Goldman-Rakic, 1991]. Whether the D5 receptor protein exists in these
cell bodies, their axonal terminals, or dendritic terminals has yet to be established. While D5 message was relatively abundant in all cortical areas, recent immunohistochemical studies with D5 antibodies localized the D5 receptor protein to the pyramidal cells in layer III of PFC [Bergson et al., 1995]. The exact subcellular localization was not determined. In contrast, D1-specific antibodies have been used to localize the D1 receptor protein to the dendritic spines of the pyramidal cells in layer III of PFC [Smiley et al., 1994]. 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.

Recent evidence from D1 'knock-out' mice studies, demonstrates that there is an apparent lack of D1-like binding in mouse brain homogenates [Xu et al., 1994b]. This might lead one to believe that there is no D5/D1b expression in mouse brain. However, D1 is important developmentally for nigrostriatal neurons [Gerfen et al., 1990], and it may be that D5 expression is dependent on proper D1 function. Again, it may be that D5 protein is present in such low quantities that radioligand binding techniques lack the sensitivity to accurately measure D5. The other alternative, that the D5 gene may not produce functional protein, is unlikely for several reasons. First and foremost, the human D5 gene has a large open reading frame that encodes for a large polypeptide, similar in structure to many other G-linked receptor proteins [Sunahara et al., 1991]. A gene with a seemingly, uninterrupted open reading frame suggests that it is functional. A gene that is not functional (i.e. not needed by the living organism) would be under no pressure not to mutate [Bailey et al., 1991]. Therefore, one would expect that a gene that is of no use to an organism would contain arbitrary mutations that would lead to premature translational stop signals and/or frame-shifts. However, Manrow et al., [1992] argue that the human prothymosin α gene has at least four pseudogenes that lack deleterious mutations within their coding regions and yet, apparently are not transcribed. Secondly, the D5 gene has, what appears to be a functional promoter region (i.e. one capable of driving transcription). This is evident by
the in vitro data presented in this thesis using various D5 promoter-luciferase chimeras, and by the presence of D5 mRNA in human and monkey brain. Presumably, genes that are transcribed are also translated into proteins.

Why then, is there a gene that encodes a protein with an almost identical pharmacological profile and relatively similar neuroanatomical distribution as another gene? The answer to this may be three-fold. The first explanation would be that the neuroanatomical distribution, while showing a great degree of overlap, is not the same. It would appear that the body needs D1-like receptors in different regions (i.e. the substantia nigra and the striatum), but this requires different molecular signals. This required diversity could potentially be achieved by having two different genes capable of responding to different intracellular signals but producing similar proteins. Furthermore, overlap or redundancy of proteins with similar functions may simply reflect a safeguard to insure the organisms survival should one system break down. Secondly, the different D1-like proteins may be needed at different stages of development. The lack of D1-like binding in D1 'knock-out' mice might suggest that D5 is expressed after D1 and is dependent on D1 expression or proper neural formation [Xu et al., 1994]. Finally, the D1 and D5 receptors do not have identical affinities for dopamine (the endogenous ligand, and therefore, the only important one, from the body's perspective). The Ki for dopamine to displace [3H]-SCH23390 at the D5 receptor is approximately 230 nM, a value that is approximately 10-fold lower than at the D1 receptor [Sunahara et al., 1991]. The in situ evidence presented in this thesis demonstrates the presence of D5 receptor mRNA in the pars compacta of the substantia nigra implicating the D5 receptor as an autoreceptor. It may be that D5, as an autoreceptor, requires a higher affinity for dopamine than does the D1 receptor which is exposed to higher concentrations of dopamine in the synapse.
3. **Functional Analysis of the D5 Gene Promoter Region**

   i.) **Transient Transfection of D5-Luciferase Constructs** - As mentioned above, the cloned D5 gene promoter sequences were fused into the luciferase gene-containing plasmid PL(KS)b-LUCnPL to demonstrate that this 5'-UTR of the D5 gene could drive transcription in the presence of the necessary nuclear factors. D5 promoter activity displayed a cell-specific pattern of expression. The results of the luciferase assays and RT/PCR assays using RNA derived from different mammalian cell lines are presented in Table 5. Seven different mammalian cell lines were tested for luciferase activity using the 5 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, indicating that these cell lines do not express the proper nuclear factors responsible for the cell-specific expression of the D5 gene product. RT-PCR analysis failed to detect the presence of D5 messenger RNA in all cell lines tested (see Table 5), and radioligand binding assays did not reveal the presence of a D1-like receptor population in the SK-N-SH cell line (data not shown). The SK-N-MC human neuroblastoma and NS20Y mouse neuroblastoma cell lines have been shown to express the D1 receptor [Gupta and Mishra, 1993; Minowa, M., et al., 1992; 1993]. NS20Y cells were not available to us. The apparent lack of D5 message, and D5 promoter-mediated luciferase activities in the SK-N-MC cell line suggests that, while the D1 and D5 receptors are very similar in gene structure and pharmacological profile, they differ greatly in the set of controls that govern their expression.

While the RT-PCR data of the immortal cell lines indicate that the SK-N-SH and GH3 cell lines did not express the D5 gene product, these cell lines appeared to contain the required factors necessary to interact with specific sequences within the D5 promoter
deletion mutants to enhance the transcription of the luciferase gene. These data could possibly indicate that an important regulatory element required for proper D5 gene expression was not present in the promoter mutants tested. A nearly ubiquitously expressed repressor protein capable of interacting with sequences either upstream or downstream from the promoter regions tested could be responsible for the rare and discrete pattern of expression for the D5 gene. The relatively low abundance of D5 message (caused by the great degree of cell specificity) may also explain the difficulty identifying a cell line that expressed the D5 gene product. 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 and Palkovits, 1992].

The D5 gene's promoter region contained several consensus sequences for multiple transcription factors. With the exception of the putative glucocorticoid response element, these consensus sequences lay within regions demonstrated, by deletion mutation analysis, to contain either positive or negative modulatory elements. For this reason, these motifs were targetted for further study using various transcriptional inducers and EMSA.

ii.) **Phorbol Ester and Glucocorticoid Effects on D5-Mediated Luciferase Activity** - The presence of a putative glucocorticoid response element (GRE) and activator protein-1 consensus sequences (AP1-a and AP1-b) in the D5 gene promoter suggested that D5 gene transcription was inducible. In order to ascertain if the D5 gene promoter was responsive to external stimuli, D5 promoter-luciferase constructs were transiently transfected into SK-N-SH and GH3 cells in the presence or absence of forskolin, or the phorbol ester TPA. In each case no significant alteration in the D5
promoter-mediated luciferase activity was observed. These data would suggest that the DNA sequences in question are not response elements at all.

Glucocorticoid responsiveness was examined in the rat osteosarcoma cell line ROS 17/2.8, which expresses high levels of glucocorticoid receptor [Rodan and Rodan, 1986]. Again, no significant increase in D5 gene promoter-mediated luciferase activity was observed. The sequence 'TGTTCT' in the D5 promoter was a 'perfect' GRE half-site, but the sequence immediately 5' to this half-site was a comparatively weak consensus sequence. However, half-sites for the estrogen, thyroid hormone and retinoic acid receptors have been shown to be functional in the rat oxytocin promoter [Adan et al., 1993]. Furthermore, accessory factor binding has been shown to be necessary for the proper glucocorticoid response in the phosphoenolpyruvate carboxykinase gene promoter [Mitchell et al., 1994], demonstrating that some genes are under a complex set of regulatory controls working through composite response elements.

The role that the AP1 consensus sequence plays is less clear. AP1 (and AP1-like) binding activity is reported to increase in the presence of sera-containing media as well as other factors such as nerve growth factor and the calcium ionophore A23187 [Cohen and Curran 1988; Franza et al., 1988; Rauscher et al., 1988]. The lack of induction seen in this study may be due to a high basal AP1 activity that any further increase may not be observable. This, however, posed a technical problem. Either the concentration of lipofectamine™ and/or the lack of sera was considerably toxic to the SK-N-SH and GH3 cells. Attempts to perform the luciferase assays in the absence of media resulted in the loss of approximately 90% (greater than this, in the case of the GH3 cells) of the cell populations. A loss of this large proportion of the cell populations resulted in an unreliable signal in the luciferase assays. Gel-shift experiments, on the other hand suggest a possible involvement by AP1 or an AP1-like protein. Specific protein-DNA complexes were observed using the GS-4 probe containing the D5-AP1 consensus sequences.
Furthermore, it appeared that the oligonucleotides containing AP1 consensus sequences reduced the intensity of at least one of these specific complexes (see Figure 36). However, the similar retardation pattern observed with probes incubated with SK-N-SH nuclear extract, a cell line in which D5-luciferase mutants are not particularly active, suggests that other factors are likely involved in the trans-activation of the D5 gene. Another possibility, yet unsubstantiated, is the presence of another nuclear factor in the GH3 cells not present in the SK-N-SH cells evident by the presence of a high molecular weight complex seen after GS-4 probe incubation with GH3 nuclear extract but not with SK-N-SH nuclear extract. However, this signal was very weak and could not be visualized after photographic reproduction of autoradiograms. Repeated attempts to get a stronger signal were unsuccessful.

The possibility that transcription factors may potentially interact with the D5 promoter at these sites may be further complicated by the need for other requirements necessary for modulation of D5 gene transcription. TPA and dexamethasone alone, or in combination had no effect at the rat D1 gene promoter, yet the addition of 8-bromo-cAMP together with these agents caused significant induction of D1 promoter-mediated CAT activity [Zhou et al., 1992]. The existence of a composite response element and the possible inter-dependence of several factors was not addressed in this work beyond the addition of TPA and forskolin to SK-N-SH cells after transfection with the various D5 promoter-luciferase chimeras (see Figure 28).

Several neurotransmitters, including dopamine are capable of activating immediate-early genes such as c-fos, c-jun and jun-B [Robertson et al., 1992]. Fos and Jun, the protein products of c-fos and c-jun respectively, heterodimerize to form the transcription factor AP1. Presumably AP1 activation is the result of dopamine receptor mediated activation of adenylyl cyclase. The AP1 consensus sites observed in the D5 promoter are located in the positive regulatory regions as assessed by deletion mutation analysis.
However, the function of AP1 (+ve or -ve), if any at all, has not been established. AP1 has been implicated as a negative modulator for the lipid-binding protein aP2 gene [Distel et al., 1987], and this type of interaction at the D5 gene promoter would potentially provide a feedback mechanism for D5 receptor desensitization. Conversely, the possibility that AP1 exerts a positive modulatory effect on D5 gene transcription would be analogous to the positive feedback mechanism seen between cAMP and the β2-adrenergic receptor and that proposed for the D1 and substance P receptors [Collins et al., 1989, 1990; Hershey et al., 1991; Zhou et al., 1992]. The D1 receptor is rapidly desensitized upon cAMP accumulation via protein degradation [Barton and Sibley, 1990]. Increased Fos and Jun activity are events mediated by dopamine stimulated adenylyl cyclase. The activation of transcription factors by D1-like receptor stimulation may be responsible for D1 and D5 receptor resensitization.

iii.) Electrophoretic Mobility Shift Assays - Putative consensus sequences for several transcription factors were identified in the D5 proximal promoter region. Comparison of the location of these sites with the results from the deletion mutant luciferase activity analysis showed that most of these sites fell within either positive or negative modulatory regions. The putative Sp1 consensus sites (Sp1-a and Sp1-b) consensus sequences fell within the negative modulatory region identified. These sites were attractive subjects of investigation because of the resemblance of this region to that of a negative modulator region identified in the rat D2 gene promoter [Minowa, T. et al., 1992, 1994]. Both the site termed Sp1-a in the D2 promoter and the site termed Sp1-a in the D5 promoter were directly 3' to a 'TG'-rich region. Nuclear proteins from NB41A3 cells bind to the Sp1 site as well as the TGGG repeat in the D2 promoter negative modulatory region. Both D5 and D2 are expressed in the pars compacta of the substantia nigra (as well as other brain regions), and could possibly be under the control of similar
regulatory mechanisms. In fact, the GH3 cell line expresses D2 receptors, however, it appears that while D5 promoter-mediated luciferase activity is high, D5 mRNA could not be detected by RT-PCR. While specific binding was demonstrated in this region of the D5 promoter, Sp1 did not appear to be the factor in question as was evident by the lack of competition with Sp1 consensus oligonucleotides.

Specific DNA-protein complexes were also observed within the regions demonstrated to be positive regulatory elements. The most proximal promoter region contained the AP1 sites and the Pit-1 site. A specific band(s) seemed to be decreased in hybridisation intensity in competition experiments using cold oligonucleotides containing the Pit-1 consensus sequence. However, these complexes were visible after incubation with SK-N-SH nuclear extracts, a non-pituitary derived cell line, in which the D5 promoter deletion mutants did not express of a very powerful positive modulatory element within this region (see Figure 27). As yet, there has been no report of D5 gene expression in the pituitary [see Moore and Lookingland, 1995] and Pit-1 is considered not to be expressed outside the pituitary gland. However, dopaminergic innervation to the pituitary is thought to down regulate the production of prolactin via activation of D2 receptor populations. Furthermore, Pit-1 is a strong transcriptional activator at the prolactin gene promoter [Nelson et al., 1988]. A Pit-1-mediated increase in D5 receptor gene expression in the anterior pituitary would provide a model for another level Pit-1-mediated control of prolactin production via dopamine stimulation. Conversely, D5 receptor stimulation could enhance the D2 effect should D5 and D2 be co-expressed in the same cell body and interact in the synergistic 'link' fashion proposed by Seeman et al. [1994]. The Pit-1 DNA recognition sequence is an 'AT' rich element. Several other POU-domain transcription factors are expressed in the brain and bind to similar motifs [Ingraham et al., 1990; Faisst and Meyer, 1992]. The D5 gene promoter may be a target for one or more of these neural-specific POU-domain proteins.
The most distal positive modulatory region also appeared to specifically bind nuclear factors. Again, the binding interactions of this specific complex were not inhibited by the commercially available oligonucleotides harboring various cis-acting elements used in competition EMSA experiments. Moreover, this region did not contain any sites highly homologous to cis-acting elements and was conspicuous only by the presence of the TC' dinucleotide repeat. DNA footprinting analysis would be required to identify the cis-acting element in this region of the D5 gene promoter. This region appeared to confer positive modulatory control in both the GH3 and SK-N-SH cell lines. In fact, most specific complexes formed were observed after incubation with GH3 or SK-N-SH nuclear extracts (see Figure 35).

iv.) SSCP Analysis of Genomic DNA's - Several reports have described polymorphisms within exonic sequences of the D5 receptor, but in each case linkage and association analysis failed to demonstrate any involvement with schizophrenia [Sommer et al., 1993; Sobell et al., 1995]. The presence of a small dinucleotide repeat sequence (TC<sub>13</sub>) within the D5 proximal promoter region suggested that this region might be polymorphic. The exact cis-acting elements in the D5 promoter responsible for trans-activation have not been defined. Therefore, we were interested in investigating whether or not there were allelic variations within this region of the human D5 gene, and whether these variations might affect the trans-activation of the D5 gene. Sherrington et al. [1993] have previously reported the existence of a polymorphic dinucleotide repeat near the D5 gene. In their study, linkage analysis of any of the polymorphic alleles to schizophrenia was not examined. Furthermore, the exact position of the dinucleotide repeat that they identified with respect to the D5 gene was not reported. We have extensively characterized the sequence information of the 3' and 5'-flanking regions of the D5 gene and believe that
the polymorphic dinucleotide repeat reported here is the most proximal to the D5 coding region.

Polymorphisms within the coding region of the D5 receptor gene have been reported [Sommer et al., 1993; Sobell et al., 1995]. Many of these mutations were silent and did not lead to changes in the proposed amino acid sequence of the D5 receptor protein. For those that did (four missense and one nonsense mutation) no association to schizophrenia was found. In each case the mutated form of the D5 gene product was not expressed in any in vitro system [Sommer et al., 1993; Sobell et al., 1995] to be tested for functional changes. Presumably, mutations that lead to missense changes in highly conserved amino acids, or those critical for ligand binding or receptor/effector coupling, could lead to a loss of activity of the protein, but no such changes have been observed. Similarly, a change in the nucleotide sequence of a cis-acting element within a gene promoter could have consequences for the transcription of that gene and the expression of its protein product. The increase or decrease in the number of nucleotides between the half-sites in estrogen, thyroid and vitamin D3 responsive elements can alter the ability with which a given factor can bind or change its receptor selectivity [Umesono et al., 1991]. Similarly, a single nucleotide substitution in the cis-acting element for the erythroid transcription factor GATA1 in the Duffy antigen/chemokine gene promoter abolishes the expression of this G-protein linked receptor [Tournamille et al., 1995]. Should a transcription factor bind within the D5 promoter's dinucleotide repeat region (i.e. have a requirement for either a 'TC'-repeat, or a pyrimidine-rich region), our data showed that the loss or gain of one 'TC'-repeat when at least 12 other repeats are present may be of no functional consequence.

This region does not contain consensus sequences for any well characterized transcription factors. A greater decrease or expansion in allele size may, however, affect the trans-activation mediated by factors binding within this region. Examination of this
region in a larger population is merited and we are currently attempting to identify the sequences within this region that are important for trans-activation.
VI. Conclusions

None of the data presented in this thesis supports or refutes either the classical or neo-classical dopamine hypothesis of schizophrenia. What is presented here places the D5 receptor at the 'scene of the crime'. The presence of D5 receptor message in discrete cortical areas and the pars compacta of the substantia nigra makes it a likely candidate for involvement in the dopaminergic dysfunction seen in various psychomotor disorders, such as schizophrenia. Furthermore, I have shown that the D5 promoter is capable of driving transcription in a heterologous reporter gene system. This provides further evidence for the existence of a dopamine D5 receptor protein in vivo. Withstanding any immunohistochemical/protein purification data this is the best evidence yet, that the human dopamine D5 gene produces a functional protein. Many of the specific aims of this project were obtained and the specific conclusions reached are summarized below.

1. The human dopamine D5 receptor gene sequence diverges from that of its two closely related pseudogenes approximately 1.9 kb 5' of the coding region. An Alu-mediated DNA recombination event may likely have been the cause for the duplication event that gave rise to the first pseudogene.

2. In situ hybridization analysis reveals a rare and cell-specific expression pattern. Highest levels of D5 mRNA are seen in the dentate gyrus and cortical layers II, IV and VI. Cell bodies in the pars compacta of the substantia nigra also appear to contain moderate levels of D5 mRNA. D5 does not appear to be abundant in 'classical' D1 receptor-enriched brain regions, such as the caudate, putamen, globus pallidus and nucleus accumbens.

3. Complementary DNA clones derived from expression studies in COS-7 cells suggest that the D5 gene has a variably sized intron in its 5' UTR of either 179 or 155 bp.
The transcriptional start site of the D5 gene is located -2125 bp 5' of the translational start site.

(4) The D5 gene promoter has neither CAAT nor TATA motifs. The D5 promoter is capable of driving the transcription of heterologous luciferase constructs in SK-N-SH human neuroblastoma cells and GH3 pituitary cells. The D5 promoter is not responsive to steroid hormones or phorbol esters. There is at least one negative modulatory region between nucleotides -500 and -251 and at least two enhancer regions between nucleotides -182 and -51, relative to transcriptional start site.

(5) The human dopamine D5 receptor gene promoter contains a polymorphic microsatellite within a region that contains a positive enhancer element. The wild-type form of this region contains 13 'TC' repeats. Two individuals, one schizophrenic, the other suffering from Huntington's Chorea had only 12 repeats, while another schizophrenic had 14 repeats. The presence of either 12 or 14 repeats does not seem to significantly alter D5 promoter-mediated luciferase activity in SK-N-SH cells.
VII. Future Studies

The work described in this thesis lays the groundwork for future studies concentrating on the signals that control D5 gene expression. The identification of possible cis-acting elements such as Sp1, AP1 and Pit-1, did little to provide information concerning the transcriptional control of D5 gene expression. DNA footprinting can be employed to determine exact cis-acting sequences and potentially yield clues to the identity of the exact transcription factors involved. Furthermore, the relative contribution to D5 gene activation by Pit-1 and/or AP1 remains to be determined. Once conditions are optimized for EMSA reactions using GS-4 as a probe, anti-Pit-1 antibodies supplied to us by Dr. Simon Rhodes of the University of California, San Diego, can be employed in 'supershift' experiments to ascertain unequivocally if Pit-1 interacts with the D5 gene promoter. Moreover, in vivo footprinting using ligation-mediated-PCR can determine if the binding of any give element identified, is a positive or a negative modulator. This method has been employed to directly demonstrate that the binding of the aryl hydrocarbon receptor complex (AhR) facilitates the binding of RNA polymerase II to the transcriptional initiation site of the cyp 1A1 gene promoter [Okino et al., 1995].

The ability of the D5 promoter deletion mutants tested, to increase luciferase gene expression in cell lines that do not express the D5 receptor protein indicates that factors that do not interact within the promoter region tested are essential factors required in the proper expression of the D5 gene. The identification and characterization of these factors would require the cloning and deletion mutation analysis of larger DNA fragments from the D5 5'-flanking region, both upstream and downstream of the transcriptional initiation site. Identification of +ve and -ve modulators responsible for cell-specific expression of D5 in human tissues could be facilitated using DNA hypersensitivity assays, that would allow investigators to look at large stretches of DNA for possible regulatory elements. Moreover, it would appear that one vital requirement for any such study would be the identification of
mortal cell lines that endogenously express the D5 gene. Lacking information on the flanking regions of D5 gene orthologues in lower species would limit this search to human-derived cell lines.

The polymorphic nature of the dinucleotide repeat reported here appeared to be of little functional consequence when tested in the heterologous luciferase assay system. However, if the 'TC' repeat region is important for D5 gene transactivation, a larger expansion or diminution of this region could be of functional consequence. A broader population study of this region in the D5 gene promoter would indicate if other polymorphic alleles exist.

Finally, we know have a tool for the localization of D5 receptor gene message. The D5-specific riboprobes used in these studies proved to be sensitive and reliable. As yet there have been few reports concerning D5 receptor gene expression in the periphery [Mezey and Palkovits, 1992; Takahashi et al., 1992]. Furthermore, characterization of D5 receptor gene message in disease brain has not yet been studied. The D5 gene must be considered to be a candidate for involvement in several neuropsychiatric disorders such as Parkinson's and schizophrenia and D5 receptor gene expression in these disorders warrants investigation.
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