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Molecular cloning and characterization of the mouse dopamine D-3 receptor gene: An additional intron and an mRNA variant

Fu, Dingyi, Ph.D.

City University of New York, 1994

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Molecular Cloning and Characterization of The Mouse Dopamine D-3 Receptor Gene: An Additional Intron and An mRNA Variant

by

Dingyi Fu

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1994

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This manuscript has been read and accepted by the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

6/6/94

Date

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Abstract

Molecular Cloning and Characterization of The Mouse Dopamine D_3 Receptor Gene: An Additional Intron and An mRNA Variant

by

Dingyi Fu

Advisor: Professor Nikolaos K. Robakis, Ph.D.

The intron-exon organization for the mouse dopamine D_3 receptor gene was investigated by molecular cloning and sequencing. The coding region of this gene spans at least 43 kilobases of the genome and is composed of 7 exons interspersed by 6 introns. A novel intron of approximately 1 kilobase in length was identified in both rat and mouse dopamine D_3 receptor genes. This intron splits former exon 4 of the rat dopamine D_3 receptor gene into two separate exons, termed exon 4 and exon 5, containing 197 and 78 nucleotides, respectively. The new intron (termed intron 4) is located between coding nucleotides 723 and 724 of the murine D_3 cDNA and corresponds by position and size to intron 5 of the dopamine D_2 receptor gene. Thus, the coding regions of the dopamine D_2 and D_3 receptor genes contain an identical number of exons (seven exons), have a very similar genomic organization and share a high degree of nucleotide homology, strongly suggesting

that both genes have a common phylogenic origin and that their divergence may result from a relatively recent evolutionary event. Besides, an intron analogous to the murine intron 4 was detected in the human dopamine D_3 receptor gene.

The entire coding nucleotide sequence, including all intron-exon junctions, of the mouse dopamine D_3 receptor gene was determined. This receptor shares a 94.4% homology with the rat D_3 receptor at the coding nucleotide level, while their homology at amino acid level is 96.9%. The intron-exon junctoins of both receptor genes occur at identical positions.

Reverse transcription-polymerase chain reaction experiments revealed a short form of mouse dopamine D_3 receptor mRNA (termed D_{3Short}) which predicts that the encoded protein lacks 21 amino acids from the putative third intracellular loop of the long form of the D_3 receptor. Sequence analysis suggested that D_{3Short} arised from a splicing event occured at an internal acceptor site within exon 6, resulting in the elimination of the first 63 nucleotides from this exon. No dopamine D_3 receptor mRNA variants were found deriving from alternative splicing of exon 5, although its counterpart, the cassette exon 6 in the dopamine D_2 receptor gene, is alternatively spliced to give rise to the D_{2Short} isoform. These data suggest a similarity in gene structure for the dopamine D_2 and D_3 receptors but a diversity at the post-transcriptional level during pre-mRNA splicing.

Acknowledgements

To my mentor Professor Nikolaos K. Robakis, Ph.D., I am much grateful for his guidance, encouragement and support which have made it possible for this work to be completed.

Sincere acknowledgements go to my Advisory Committee members, Dr. James L. Roberts, Dr. Jurgen Brosius, Dr. Catherine Mytilineou-Knight and Dr. Emmanuel Landau, for their invaluble advices. I also thank Dr. Bernard Cohen and Dr. Gerald Cohen who showed me a broad avenue in neurosciences and an interesting field of dopamine neurotransmission, and Dr. Beth S. Schachter who incited my desire to pursue studies in molecular biology.

I acknowledge The Graduate School of Biomedical Sciences, The Mount Sinai School of Medicine and The City University of New York who provided me with facilities and fundings towards my scientific pursuits. I thank all the teachers, officers and co-workers.

All the past and current members of Dr. Robakis' laboratory were a great pleasure to work with. I would like to extend special thanks to Dr. Lawrence M. Refolo, Ms. Michele Mohamadi, Dr. Kumar Sambamurti and Dr. Boris Skryabin for their pleasant cooperation and many helps.

Finally, I must thank my family and friends for their love and friendship over the past years.

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PART ONE: Background Overview

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9A. Significance of Neurotransmission

One of the most striking characteristics of the nervous system is the sophisticated machinery for both the speedy electric pulse transduction on the cell membrane and the various synaptic neurotransmissions between neuronal cells. This machinery allows the efficient control of the body's physical as well as mental activities. Nervous pulse transduction provides rapid transmission of action potentials along the surface of the neurons, mainly along the axons, in a fraction of a second, allowing the body's quick responses to the environmental and internal stimulations. Neurotransmission, on the other hand, involves the procedures of biosynthesis of chemical transmitter, synaptic transmission and effects on postsynaptic membrane-bound receptors. Every step of these procedures is tightly controlled and regulated. Furthermore, the idea "one neuron one transmitter" has been revolutionized since many neurons have been found to synthesize and use more than one neurotransmitter. Similarly, one neurotransmitter can often triger various cellular responses via different receptor subtypes and different intracellular signal transduction systems. The functional complexity of the nervous system relies upon these meticulous and exquisite machineries.

Neurotransmission is also involved in the pathology of many neuropsychiatrical diseases. For instance, Parkinson's disease stems from the degenerative changes of the dopaminergic neurons in the substantia nigra region

of the brain, which subsequently impair dopamine neurotransmission in the basal ganglia (Kopin, 1994). There is an abnormal elevation of dopamine receptors in patients with schizophrenia (Lee and Seeman, 1977; Lee and Seeman, 1980; Seeman *et al.*, 1993). Amphetamine, known as a dopamine agonist, can produce psychotic states resembling paranoid schizophrenia (Angrist and Gershon, 1970) and exacerbate existing symptoms in schizophrenic patients (Janowsky and Davis, 1976). Antipsychotic drugs or so-called neuroleptics are a group of dopaminergic antagonists which have action sites on certain types of dopamine receptors (Carlsson and Lindqvist, 1963; Seeman *et al.*, 1975; Seeman, 1987; Owen and Cross, 1989; Seeman, 1993). Investigation on dopamine neurotransmission and dopamine receptors will help elucidate the etiology of schizophrenia and benefit the development of more effective neuroleptics.

9B. Dopamine and Dopaminergic Projection

Dopamine was first identified as an intermediate substance in the biosynthetic pathway of two other catecholamine neurotransmitters, norepinephrine and epinephrine, using dietary tyrosine as the starting substrate (Figure 9-1). Ever since 1960's, dopamine has also been recognized as a biosynthetic terminal substance in some neurons of the brain and sympathetic ganglia, acting as an important neurotransmitter. Now, the dopaminergic neurons, together with their different receptor subtypes, have been established as an independent

neurotransmission system, anatomically and functionally distinct from those of other catecholamines and other neurotransmitters.

The human brain contains approximately 400,000 dopaminergic neurons which is a much larger number than those of either norepinephrine or epinephrine neurons (Webster, 1989). Most of the dopaminergic cell bodies are located in the midbrain. Eighty percent of them are clustered in the zona compacta of the substantia nigra (designated as area 9 or **A9**) and the retrorubral nucleus (**A8**), with their axons projecting mainly to the dorsal striatum (caudate nucleus and putamen), which is called **mesostriatal** or **nigrostriatal system**. Some dopaminergic neurons originating from the ventral tegmental area (**A10**) send their axons more medially to the ventral striatum (nucleus accumbens and olfactory tubercle) and to the limbic structures (septum and amygdala), collectively forming so-called **mesolimbic system**. Other dopaminergic fibers from A10 project to the broad limbic cortex (medial prefrontal, pyriform, cingulate and entorhinal cortices), being termed **mesocortical system.**

Some non-mesencephalic dopaminergic neurons situated in the hypothalamic and periventricular nuclei projecting to the median eminence and pituitary are responsible for some neuroendocrine activity such as prolactin release (Webster, 1989). Still other dopaminergic neurons with short axons are found within the olfactory bulb and retina (Cooper *et al.*, 1991; Lacey, 1989; Robbins, 1992). Figure

9-2 illustrates the general organization of the main dopaminergic projections.

The striatum is the major integrative center of the basal ganglia. Together with its inward and outward fibers, it composes the extrapyramidal system. Surgical or pharmacological (such as MPTP-intoxication) deletion of dopaminergic innervation from the striatum results in deficiency and imbalance of voluntary movement mimicking the symptoms of Parkinson's disease (Cooper *et al.*, 1991; Kopin, 1994).

The mesolimbic system of the dopaminergic neurons is thought to play a critical role in locomotion, motivation and behavioral reward. With the participation and coordination of the mesocortical system, it may also be involved in cognitive and emotional activity (Fibiger and Phillips, 1986). It has been known that chronic amphetamine self-administration produces symptoms strongly resembling some aspects of paranoid schizophrenia and this is generally thought to result from overrelease of dopamine caused by amphetamine (Ellinwood, 1967; Ellinwood, 1968). The mesolimbic and mesocortical target areas are considered to be the action sites of most neuroleptic drugs used for treatment of schizophrenia and other psychiatric disorders (Owen and Cross, 1989).

9C. The Dopamine Receptor Family

Dopamine neurotrasnsmission is mediated by postsynaptic dopamine receptors. Five subclasses of dopamine receptors have been cloned so far and they are categorized, according to their molecular structure and biochemical and pharmacological characteristics, as dopamine **D_1-like** (D_1 and D_5) and **D_2-like** (D_2, D_3 and D_4) receptor subfamilies. The dopamine D_1 receptors were first found in the brain and parathyroid gland but were absent from the pituitary. They are functionally associated with stimulation of intracellular adenylate cyclase. The dopamine D_2 receptors were first found in the brain and pituitary but were absent from the parathyroid gland, and are either inhibitory to adenylate cyclase or independent of this enzyme (Niznik, 1987). Ligand binding experiments show that dopamine D_1 and D_2 receptors have different agonists and antagonists (Table 9-1). However, the behavioral effects of neuroleptic agents appear to be mostly mediated via the D_2 receptors. It has long been noticed that there is an elevation of D_2, but not D_1, receptor density in the striatum and nucleus accumbens in untreated patients with schizophrenia (Seeman, 1993).

The dopamine D_1 and D_2 receptors are widely distributed in both the basal ganglial region and the limbic-cortical areas, with the highest levels of expression being observed in the caudate and putamen, nucleus accumbens and olfactory tubercle. *In situ* hybridization studies showed that both D_1 and D_2 mRNAs have

largely overlapping yet distinct patterns of expression (O'Dowd, 1993). For instance, while the medium-sized neurons (10-15 μm) in caudate and putamen expressed both mRNAs, only the D_2 mRNA is observed in the larger neurons (>20 μm). Within the neocortex, D_1 mRNA is observed primarily in layer 6 (Dawson *et al.*, 1986) while D_2 mRNA is found primarily in layers 4 and 5 (Bouthenet *et al.*, 1987). Besides, only D_2 mRNA was detected in substantia nigra where most dopaminergic cell bodies are located (Weiner *et al.*, 1991), suggesting that D_2 receptors also exist as autoreceptors.

The dopamine D_3 receptors are mostly expressed in the limbic area (heavily expressed in the Islands of Calleja, nucleus accumbens and amygdala) and the cerebral cortex including the hippocampus (Sokoloff *et al.*, 1990). In *in vitro* expression systems, the D_3 receptors displayed high affinities for both dopamine itself and most neuroleptics (Sokoloff *et al.*, 1990). Relatively high levels of the dopamine D_4 receptor mRNA were observed in the frontal cortex, midbrain area, amygdala and medulla, but low levels were found in the basal ganglia (Van Tol *et al.*, 1991). The D_4 receptors share some pharmacological characteristics with D_2 and D_3 receptors, and display a high affinity specifically for the antipsychotic drug clozapine which, in contrast to most other drugs, does not cause side effects such as tardive dyskinesia (Van Tol *et al.*, 1991). The dopamine D_5 receptors are expressed in the frontal cortex, striatum, hippocampus and hypothalamus, and the abundance of D_5 mRNA is about one order of magnitude lower than that of D_1

mRNA (Sunahara *et al.*, 1991). Table 9-2 summarizes these five subtypes of cloned dopamine receptors.

9D. Molecular Structure of The Dopamine Recptors

The dopamine receptors are all integral membrane proteins and their molecular structure is very similar to each other. Hydropathy analysis reveals that these monomeric proteins have typical structural characteristics which belong to the superfamily of regulatory GTP-binding protein (G protein)-linked receptors including rhodopsin (so-called light receptor), the muscarinic acetylcholine receptor and adrenergic receptor (O'Dowd *et al.*, 1991; Strosberg, 1991). Each of these receptors contains seven hydrophobic *trans*-membrane (TM) α-helical domains (each TM helix is 20-25 amino acids long) with the N-terminal domain protruding extracellularly and the C-terminal domain intracellularly. In the three-dimensional model, the seven TM helices of the dopamine receptors are so bundled as to form a binding pocket (or a cavity) which leaves space in the cell's lipid bilayer membrane for the binding of dopamine agonists and antagonists (Strange, 1990). The ligand binding sites of the dopamine receptors are believed to be provided by the specific amino acid side-chains on the TM helices 3 and 5 which face to the cavity (Strange, 1990; O'Dowd *et al.*, 1994). The dopamine receptors, like other G protein-coupled receptors, are glycoproteins containing *N*-linked sugars on the extracellular domains. In addition, they are phosphorylated at several sites of the

putative third intracellular loop and/or the C-terminal cytoplasmic tail, which contain consensus sequences for phosphorylation (Weiner and Molinoff, 1994). These intracellular regions are also considered important for the coupling of dopamine receptors to G proteins during intracellular signal transduction (Strange, 1990; O'Dowd, 1993).

Molecular cloning and characterization of dopamine receptors have been used to provide a powerful means to study their structure and function. The dopamine D_1 receptor was deduced to contain 446 amino acids in rat, mouse and human (Dearry *et al.*, 1990; Zhou *et al.*, 1990; Sunahara *et al.*, 1990). The structure of this molecule closely resembles that of the β_2 adrenergic receptor with a relatively short third intracellular loop and a long carboxy tail (Figure 9-3). This structure is a characteristic of receptors coupled to G_s proteins and involves the activation of adenylate cyclase (Sibley and Monsma, 1992). The human dopamine D_5 receptor, the other member of the D_1-like receptor subfamily, is a 477 amino acid protein with a 50% overall, and an 80% TM-regional homology to the D_1 receptor (Sunahara *et al.*, 1991). The D_5 receptor has a very similar molecular structure with the D_1 receptor (Sibley and Monsma, 1992). The rat homologue of the human D_5 receptor was cloned and termed as D_{1B} receptor (Monsma *et al.*, 1991; Tiberi *et al.*, 1991). This rat receptor is 475 amino acids in length and is 83% identical overall but 95% identical in the TM-regions in comparison with the human D_5 receptor (Sibley *et al.*, 1994).

The human dopamine D_2 receptor protein contains 443 amino acids while the murine D_2 receptor has 444 amino acids. This receptor has a relatively large third intracellular loop and a short C-terminus (Figure 9-4), a characteristic of most receptors coupled to G_i proteins and related to inhibition of adenylate cyclase (Sibley and Monsma, 1992), such receptors include α_2 adrenergic receptor and M2 muscarinic acetylcholine receptor. The rat dopamine D_3 receptor has 446 amino acids and the human D_3 receptor has 400 amino acids. The amino acid sequence of the D_3 receptor is similar to that of the D_2 receptor, exhibiting a 52% overall homology with the D_2 receptor. The D_3 receptor shows a similar membrane topology with the D_2 receptor (Figure 9-5), and has comparable binding activities for dopaminergic ligands in *in vitro* expression systems (Sokoloff *et al.*, 1990). However, the intracellular coupling features of the D_3 receptor have not been elucidated. The dopamine D_4 receptor, the third member of the D_2-like receptor subfamily, contains 387 amino acids and shares a 41% and 39% overall homology with the dopamine D_2 and D_3 receptors, respectively (Van Tol *et al.*, 1991). It shows structural features similar to the D_2 and D_3 receptors except that its third intracellular loop domain is about one third shorter than those of the D_2 and D_3 receptors and contains several nucleotide repeat sequences in this region (Van Tol *et al.*, 1991; Van Tol *et al.*, 1992).

The amino acid sequence homology of the dopamine receptor proteins is

higher in the seven TM domains than in any other part of these receptors. The amino acid identity in TM regions is always greater than 50% between members within each dopamine receptor subfamily while it drops below 50% if compared between dopamine receptor subfamilies. For instance, the identity of TM regions is 75% between D_2 and D_3, 80% between D_1 and D_5 and 44% between D_1 and D_2 receptors (Sibley, 1991; Sibley and Monsma, 1992).

9E. Genes Encoding Dopamine Receptors

The categorization of the two dopamine D_1-like and D_2-like receptor subfamilies is further supported by the fact that they are encoded by two different gene families, *i.e.*, the D_1-like and D_2-like receptor genes. The D_1-like receptor genes have an intronless amino acid encoding region, whereas the D_2-like receptor genes contain 4-6 introns in the coding region (Figure 9-6). The dopamine D_1 receptor gene is located on human chromosome 5 at q35.1 (Grandy *et al.*, 1990) and is a single copy gene having a long open reading frame which encodes a 446 amino acid protein. Its 5' untranslated region, however, was reported to contain a small intron of 116 base pairs (Minowa *et al.*, 1992). The dopamine D_5 receptor has been mapped to human chromosome 4 at p15.1-p15.3 (Polymeropoulos *et al.*, 1991; Eubanks *et al.*, 1992). Like the D_1 receptor gene, the D_5 receptor gene is intronless in the coding region. In addition, a dopamine D_5 receptor pseudogene was reported to be present in each one of the human chromosomes 1 and 2.

These pseudogenes have a 95% nucleotide homology to the D_5 receptor sequence. Due to the existence of nucleotide insertions and deletions which alter the reading frame, the D_5 pseudogenes were reported to be merely able to direct the synthesis of an abnormally short polypeptide (Grandy *et al.*, 1991; Nguyen *et al.*, 1991a; Weinshank *et al.*, 1991; Nguyen *et al.*, 1991b).

The dopamine D_2 receptor gene is located on the q22-q23 region of the human chromosome 11 (Grandy *et al.*, 1989a) and contains 8 exons interspersed by 7 introns. Except for exon 1, all the amino acid coding exons (exons 2-8) of the D_2 gene are clustered within a stretch of 11-13 kilobases (there is a small species variation among rat, mouse and human) (Mack *et al.*, 1991). Exon 1 which contains the 5' untranslated sequence is seperated from exon 2 by an intron of at least 38 kilobases (Mack *et al.*, 1991; O'Dowd, 1993; Buck, *et al.*, 1992). Thus, the entire D_2 receptor gene spans more than 52 kilobases.

The dopamine D_3 receptor gene has been mapped on human chromosome 3 at q13.3 (Le Coniat *et al.*, 1991). Although the D_3 receptor shares higher homology with the D_2 receptor than with any other dopamine receptor, the amino acid sequence of the D_3 receptor was reported to be encoded by six exons, which differs from the number of exons encoding the amino acid sequence of the D_2 receptor (see text above and Figure 9-6). In a previous report (Fu *et al.*, 1993; Part II of this dissertation), we described the discovery of an additional intron in the D_3

receptor gene which splits former exon 4 into exons 4 and 5. Besides, a putative intron acceptor consensus sequence was found at -34 of the ATG initiation codon of the D_3 receptor gene (Giros *et al.*, 1991; Part II of this dissertation), implying that an additional intron may also be present in the 5' untranslated region (yet to be confirmed) as is the case for the D_2 receptor gene which contains an intron (intron 1) at a very similar position (-31 of the first ATG codon).

The dopamine D_4 receptor gene, the third dopamine D_2-like receptor gene, is located at the tip of the short arm of the human chromosome 11 (Petronis *et al.*, 1993). It contains 4 introns in the coding region. Interestingly, intron 3 of the D_4 receptor gene has an unusual intron-exon junction where the conventional splice sequences for donor and acceptor sites are missing; instead, a part of a repeat sequence appears in this region (O'Malley *et al.*, 1992; Van Tol *et al.*, 1992; Figure 9-6).

The putative promotor regions of dopamine D_1 and D_2 receptor genes have been analysed to some extent. They are found to be GC-rich and to contain several consensus sequences of *cis*-acting elements. However, no conventional TATA-box or CCAAT-box has been identified. These genes are considered to be housekeeping genes which, however, are expressed in a tissue-specific and regulatory manner (Minowa *et al.*, 1992a; Minowa *et al.*, 1993a; Minowa *et al.*, 1992b; Minowa *et al.*, 1993b).

9F. Dopamine Receptor mRNA Variants and Genetic Polymorphisms

Two isoforms of D_2 mRNA, D_{2Long} and D_{2Short}, have been found to derive from the alternative splicing of a small exon encoding 29 amino acids of the third intracellular loop. Both isoforms are expressed in human, rat, and mouse brain, although the long form is always the predominant one in almost all the areas detected (Mack *et al.*, 1991). No pharmacological differences have so far been observed between these protein isoforms. However, D_{2Short} protein was found to bind adenylate cyclase slightly more efficiently than D_{2Long} (Dal Toso *et al.*, 1989; Montmayeur and Borrelli, 1991; Hayes *et al.*, 1992). The 5' untranslated sequence of a human retina D_2 receptor cDNA clone (termed clone 11.1) was reported to be partially different from that in a pituitary D_2 cDNA clone, presumably resulting from alternative splicing. Another human retina D_2 receptor cDNA clone 11.2 had a slightly shorter 3' non-coding sequence than clone 11.1 (Robakis *et al.*, 1990a; Robakis *et al.*, 1990b).

Several short mRNA variants for the dopamine D_3 receptor have been described. They reflect mRNA transcripts with deletions and truncations which result from alternative splicing in the coding regions of either the putative second *trans*-membrane domain, the second intracellular loop or the second extracellular loop (Giros *et al.*, 1991; Snyder *et al.*, 1991; Nagai *et al.*, 1993). Furthermore, another rat D_3 receptor mRNA was found to contain an additional sequence of 84

nucleotides, presumably derived from an extra exon of the D_3 receptor gene (Pagliusi, 1993). However, no considerable specific dopaminergic binding activity was achieved from each of these variants in *in vitro* expression systems. Fishburn *et al.* (1993) recently described a novel mRNA variant (called D_{3Short}) which exibited binding activity for dopaminergic agonists and antagonists. This variant lacks 63 nucleotides in the region encoding the putative third intracellular loop, resulting from using an internal acceptor splicing site inside exon 6. This mRNA encodes a 425 amino acid D_3 receptor protein. As revealed by polymerase chain reaction experiments, D_{3Short} mRNA was expressed in the same brain areas as D_{3Long} mRNA although the latter was always the predominant species. However, the expression ratio of D_{3Short}/D_{3Long} receptors varied between 1:1.5 and 1:20. Northern hybridization studies further demonstrated that, like D_{3Long} mRNA, the D_{3Short} mRNA was richest in the olfactory tubercle and hypothalamus where the D_{3Short} mRNA levels were more than 10 times higher than in most of other tissues examined (Fishburn *et al.*, 1993).

The third intracellular loop of the human dopamine D_4 receptor has been reported to contain 1-7 copies of a 16 amino acid motif which derives from the repeat of a 48 basepair sequence in the D_4 receptor gene (Van Tol *et al.*, 1992). This D_4 sequence polymorphism was suggested to have genetic association with chronic alcoholism (George *et al.*, 1993), and to reflect the differences of D_4 variants in their affinities for various neuroleptics including clozapine, an atypical

neuroleptic agent (Van Tol *et al.*, 1992). Another polymorphism in the length of intron 1 of the human D_4 receptor gene was also reported (Petronis *et al.*, 1994).

It was reported that a transcript from the dopamine D_5 receptor pseudogenes was detected in many areas of the human brain, and this mRNA variant was capable of forming a 154 amino acid peptide, a truncated D_5 receptor form resulting from a premature termination of translation due to nucleotide-reading frame-shifting (Weinshank *et al.*, 1991; Nguyen *et al.*, 1991b).

Table 9-1. Dopamine receptor subtypes classified on the bases of anatomical, pharmacological and biochemical studies.

	D_1	D_2
Distribution		
striatum	+	+
parathyroid gland	+	-
pituitary	-	+
Pharmacology		
selective agonists	SKF38393 fenoldopam	N-0437 bromocriptine
selective antagonists	SCH23390 SKF83566 SCH39166	(-)-sulpiride YM091512 domperidone
Effector responses		
adenylate cyclase	activation	inhibition
K^+ channel	-	activation
Ca^{2+} channel	-	inhibition

Table 9-2. Synopsis of features of cloned dopamine receptor subtypes.

	D_1-like		D_2-like		
	D_1	D_5	D_2	D_3	D_4
Number of amino acids					
mouse	446		444/415	446/425	
rat	446	475	444/415	446	385
human	446	477	443/414	400	387
Gene location on chromosome	5q	4p pseudogenes on human 2p and 1q	11q	3q	11p
Selective gene expression			D_{2Long}/D_{2Short} in mouse, rat and human, via cassette exon alternative splicing	D_{3Long}/D_{3Short} in mouse only, via internal acceptor alternative splicing	polymorphism in human at the third intracellular loop encoding region
Number of introns in coding sequence	0	0	6	6	4
Brain regions enriched*	C/P OT NA	OT, NA FCX C/P	C/P OT NA	OT NA IC	FCX AMG Midbrain
Antagonists					
prototypic	SCH23390	SCH23390	spiperone	spiperone	spiperone
selective			haloperidol	AJ76, UH232	clozapine
Predominant effector	cAMP activation	cAMP activation	cAMP inhibition	?	cAMP inhibition
G protein coupled	Gs	Gs	Gi/Go	?	Gi/Go

*Abbreviations: C/P=caudate/putamen, OT=olfactory tubercle, NA=nucleus accumbens, IC=islands of Calleja, FCX=frontal cortex, AMG=amygdala.

Figure 9-1. Biosynthetic pathway for catecholamine neurotransmitters. (page 20)

The catecholamine neurotransmitters comprise dopamine, norepinephrine and epinephrine. They share the same biosynthetic pathway but their corresponding neurons have different final products. (Reprint with permission, Weiner, N. and Molinoff, P.B., 1989)

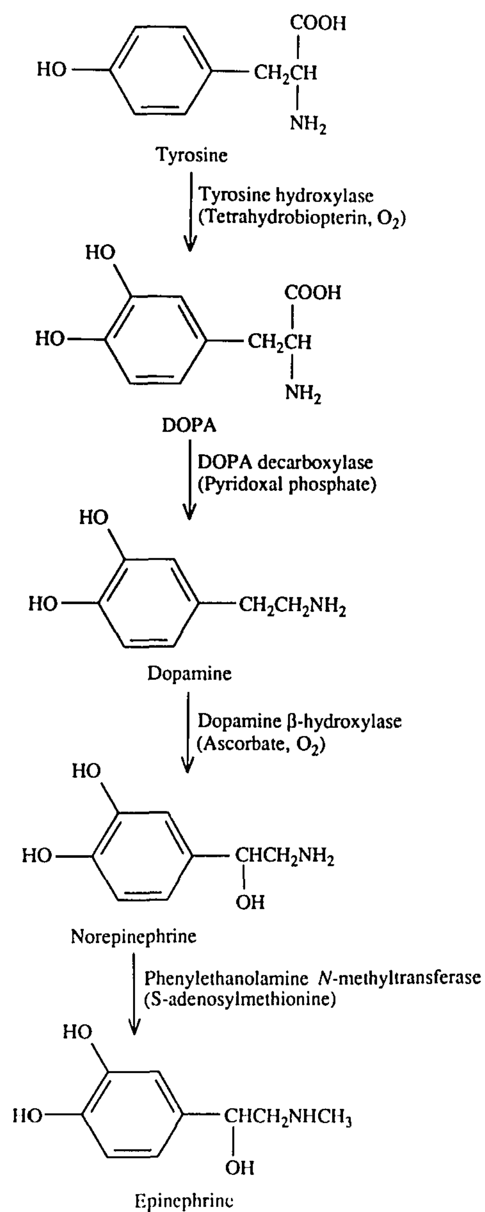
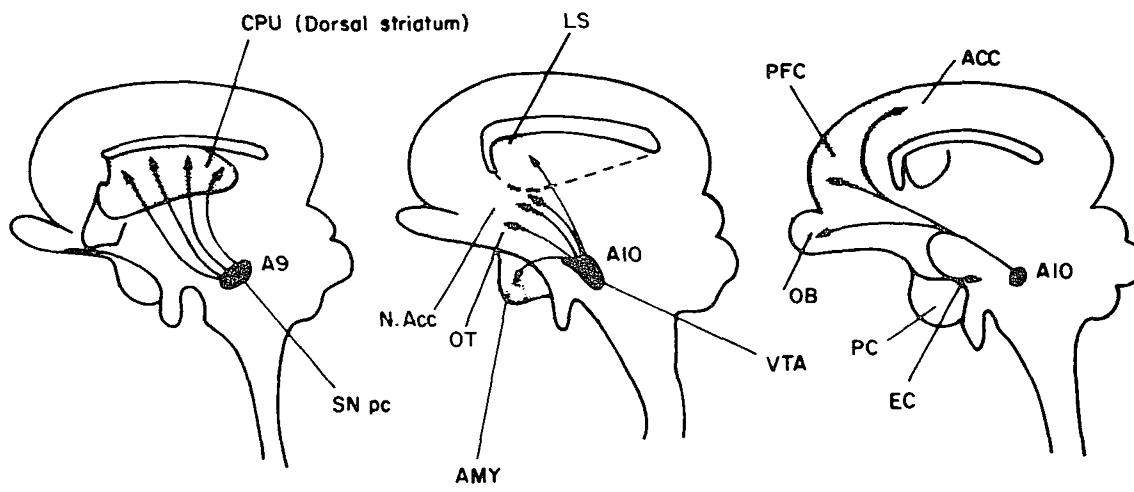

HO
COOH
CH2CH
NH2
Tyrosine
Tyrosine hydroxylase
(Tetrahydrobiopterin, O2)
HO
HO
COOH
CH2CH
NH2
DOPA
DOPA decarboxylase
(Pyridoxal phosphate)
HO
HO
CH2CH2NH2
Dopamine
Dopamine β-hydroxylase
(Ascorbate, O2)
HO
HO
CHCH2NH2
OH
Norepinephrine
Phenylethanolamine N-methyltransferase
(S-adenosylmethionine)
HO
HO
CHCH2NHCH3
OH
Epinephrine

Figure 9-2. Schematic diagrams of the main dopamine systems in sagittal sections of the brain. (page 22)

The main dopamine systems comprise the mesostriatal, mesolimbic and mesocortical systems. Shaded areas indicate regions of innervation. Abbreviations: A9, cell group of dopamine neurons in the substantia nigra, pars compacta (SNpc) of the mesencephalon; A10, cell group of dopamine neurons in the ventral tegmental area (VTA) of the mesencephalon; CPU, caudate and putamen; N.Acc, nucleus accumbens; OT, olfactory tubercle; AMY, central amygdaloid nucleus; LS, lateral septum; OB, olfactory bulb; PFC, prefrontal cortex; ACC, anterior cingulate cortex; PC, pyriform cortex; EC, entorhinal cortex. The A8 cell group of dopamine neurons in the retrorubral nucleus is not shown here. (Reprint with permission, Robbins, T.W., 1992)



A) Mesostriatal B) Mesolimbic C) Mesocortical

Figure 9-3. Model of the human dopamine D_1 receptor. (page 24)

The shaded area represents lipid bilayer membrane. The N-terminus is extracellular. Potential glycosylation sites are indicated as Y. A cysteine residue found in the C terminal part of the receptor molecule has been placed close to the lipid bilayer to indicate its possible anchoring role through palmitoglation. The hexagonal cysteine residues might form disulfide bonds. The black residues indicate putative phosphorylation sites by cAMP-dependent protein kinase A. The square and triangular amino acid residues are expected to be involved in ligand binding. (Reprint with permission, Civelli, O. *et al.*, 1991)

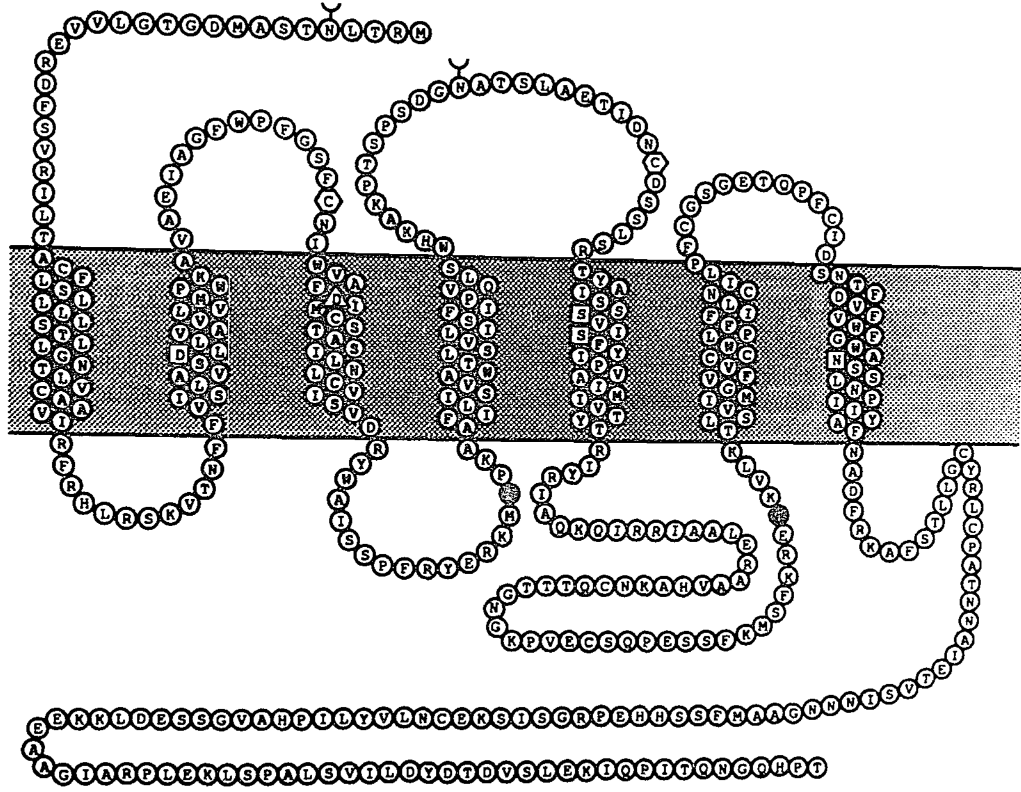
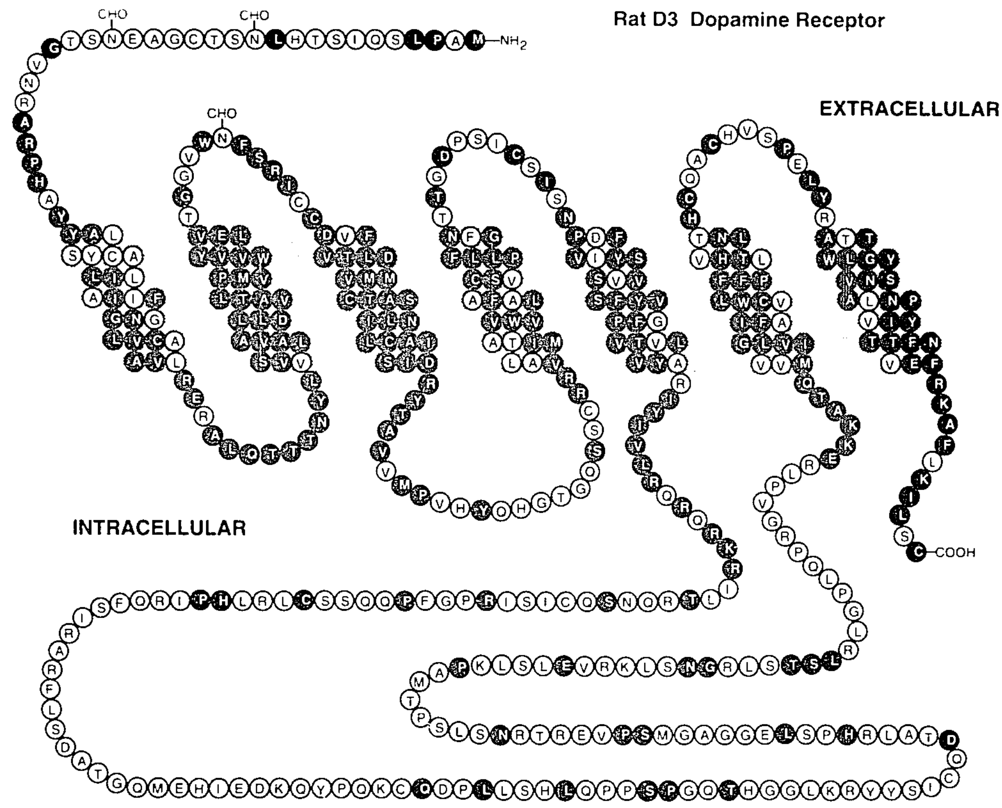


Figure 9-4. Model of the human dopamine D_2 receptor. (page 26)

The shaded area represents lipid bilayer membrane. The N-terminus is extracellular. Potential glycosylation sites are indicated as Y. The last cysteine residue on the C termius has been placed close to the lipid bilayer to indicate its possible anchoring role through palmitoglation. The hexagonal cysteine residues might form disulfide bonds. The black residues indicate putative phosphorylation sites by cAMP-dependent protein kinase A. The square and triangular amino acid residues are expected to be involved in ligand binding. The shaded amino acid residues in the third intracellular loop are the part resulting from alternative splicing event. (Reprint with permission, Civelli, O. *et al.*, 1991)

Figure 9-5. Model of the rat dopamine D_3 receptor. (page 28)

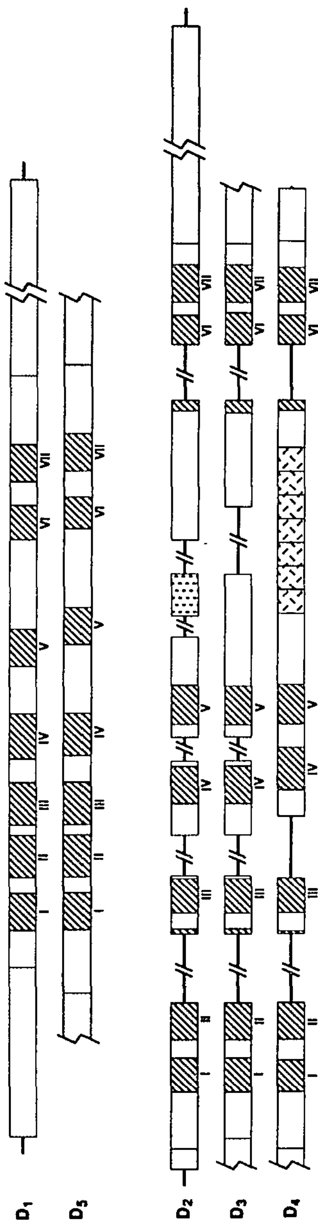
This shows the proposed membrane topography of the rat dopamine D_3 receptor and its relationship with the D_2 receptor. Membrane-spanning domains are defined on the basis of hydropathy analysis. Solid circles indicate amino acids that are identical in both the D_2 and D_3 receptors. CHO's indicate potential N-linked glycosylation sites. (Reprint with permission, Sibley, D.R., 1991)



Rat D3 Dopamine Receptor
EXTRACELLULAR
INTRACELLULAR
CHO
CHO
CHO
—NH2
—COOH

Figure 9-6. Genomic organization of the dopamine receptor genes. (page 30)

Lines indicate introns, boxes exons; striped boxes with Roman numerals show the location of the putative transmembrane domains, shaded boxes those of the untranslated region of the corresponding mRNA; the pointed exon in the D_2 receptor gene is the alternatively spliced exon differentiating D_{2Short} from D_{2Long}. The seven repeats found in some human genes are outlined in hatched boxes in the D_4 receptor gene. (Reprint with permission, Civelli, O. *et al.*, 1993)


D1
D5
D2
D3
D4
I
II
III
IV
V
VI
VII

PART TWO: The establishment of the gene structure for the murine dopamine D_3 receptor and the detection of an additional intron in the dopamine D_3 receptor gene

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10A. Introduction

In the central nervous system, the neurotransmitter dopamine and the associated dopamine receptors are of special importance for the control of body's motor function and behavioral homeostasis. Growing evidence suggests the involvement of the dopamine receptors in such neuropsychiatric and genetic events as Parkinsonism, schizophrenia, neuroleptic pharmacology, drug addiction and alcoholism (Strange, 1993; Civelli *et al.*, 1993). Until recent categorization of the dopamine D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4) receptor subfamilies, the dopamine receptors were classified as D_1 and D_2 subclasses mainly according to their anatomical, pharmacological and biochemical profiles (Creese *et al.*, 1983; Niznik, 1987). They could be prepared to purity from different tissue sources and were differentiated by their specific agonists and antagonists (Table 9-1). Further biochemical investigations showed that they were also characterized by different intracellular secondary messenger pathways. The dopamine D_1 receptors were found to be invariably associated with adenylate cyclase which they stimulate while D_2 receptors were either inhibitory to adenylate cyclase or independent of this intracellular enzyme. More recently, the D_2 receptors were also reported to exert effects on phosphatidylinositol turnover and on potassium and calcium ion channel activities via different inhibitory G proteins (Enjalbert *et al.*, 1986; Freedman and Weight, 1988; Enjalbert *et al.*, 1988; Ohara *et al.*, 1988; Elazar *et al.*, 1989; Senogles *et al.*, 1990). This biological heterogeneity of the dopamine D_2 receptors has

become greatly interesting since other D_2-like receptor subtypes and D_2 mRNA isoforms were found and cloned, among which the D_3 receptor is of special importance. Molecular cloning and characterization of the dopamine receptor subtypes provide a powerful means to study the relationship between their structure and function at the molecular level.

The dopamine D_3 receptor was first cloned from rat by screening cDNA and genomic libraries and by a combination of reverse transcription and polymerase chain reaction using information from the D_2 receptor nucleotide sequences (Sokoloff *et al.*, 1990). When expressed in Chinese hamster overy cells, the D_3 receptors showed a binding affinity for dopamine about 20 times higher than the D_2 receptors. Interestingly, many "typical" neuroleptics, a group of dopamine antagonists displaying both antipsychotic activity and extrapyramidal side effects (such as tardive dyskinesia), interact 10 to 20 times more efficiently with the D_2 receptor than with the D_3 receptor, whereas many "atypical" neuroleptics, with less side effects than the typical neuroleptics, are only 2 to 3 times more potent at D_2 sites than at D_3 sites. Some atypical agents even exhibited significantly higher potency at the D_3 receptor sites than at the D_2 sites. Besides, the anatomical distribution pattern of the D_3 receptor mRNA in the brain somewhat overlaps with, but markedly differs from, that of the D_2 receptor mRNA. For instance, only a weak D_3 mRNA signal was detected in ventral striatum, whereas the D_2 mRNA was found throughout the striatum. On the other hand, the D_3 receptor mRNA

is expressed at high levels in the olfactory tubercle-island of Calleja complex and nucleus accumbens. These areas constitute, together with ventral striatum, hippocampus, septum, amygdala and mammillary nuclei where the D_3 mRNA is also detected, a territory known as limbic area, implying an important participation of the D_3 receptor in dopaminergic transmissions connected with cognitive, emotional and endocrine functions (Sokoloff *et al.*, 1990).

The gene structure of the dopamine D_2 receptor was quite extensively investigated in rat, mouse and human (Grandy *et al.*, 1989b; O'Malley *et al.*, 1990; Mack *et al.*, 1991), and it shows a high degree of similarity in both size and structural organization among these species (See Part I of this dissertation at section 9E). Thus far, the D_3 receptor gene structure was investigated in rat (Giros *et al.*, 1991) and partially studied in human (Giros *et a.*, 1990). In order to better understand the D_3 receptor gene expression and D_3 receptor function in *in vivo* systems, transgenic experiments with reconstructed receptor DNA in animal models will be extremely beneficial. As a first stage for this purpose, we report here the elucidation of the intron-exon organization of the mouse dopamine D_3 receptor gene, and the establishment of a restriction endonuclease map which will facilitate the genomic DNA reconstruction.

While the D_1 and D_5 receptor genes are found to be intronless in their coding region like many other G protein-coupled receptor genes, each of the D_2,

D_3 and D_4 receptor genes contains several introns (Schwartz *et al.*, 1992; Civelli *et al.*, 1993; Figure 9-6). The D_2 receptor contains 444 amino acids encoded by seven exons interspersed by six introns. The region of the gene encoding the putative third intracellular loop contains two introns, one situated between coding nucleotides 723 and 724, and the other between nucleotides 810 and 811. A cassette exon of 87 basepairs flanked by these two introns is alternatively spliced resulting in the generation of two isoforms of D_2 receptor (D_{2Long} and D_{2Short}) which differ in 29 amino acids encoded by these 87 nucleotides (Giros *et al.*, 1989; Monsma *et al.*, 1989; Dal Toso *et al.*, 1989). Although the dopamine D_3 receptor shares high amino acid homology with the D_2 receptor and they are believed to have a common phylogenic origin, the rat D_3 receptor gene was reported to contain five introns along the entire 446 amino acid coding area, only one of which (between coding nucleotides 801 and 802) was found within nucleotide sequence encoding the third intracellular loop (Giros *et al.*, 1991; Schwartz *et al.*, 1992; Civelli *et al.*, 1993). Here we describe the discovery of an additional intron in the mouse, rat and human dopamine D_3 receptor genes, which is analogous to intron 5 of the D_2 receptor gene in the region coding for the putative third intracellular loop (Fu *et al.*, 1993).

10B. Materials and Methods

Genomic cloning and mapping: A mouse genomic library (129SV, in lambdaphage FIX™ II) was purchased from Stratagene, CA. To screen this library, four DNA probes (D3001, D3002, D3003 and D3005, see Table 10-1) were designed based on the rat dopamine D_3 receptor sequence (Sokoloff *et al.*, 1990; Giros *et al.*, 1991) and synthesized in a Model 380B DNA synthesizer (Applied Biosystems Inc., CA). For each probe, a half million plaque forming units from the 129SV library were plated on 150mm agar petri dishes in 1X NZYDT broth (Gibco BRL, MD) with *E. coli* strain LE392 as the host. The dishes were incubated at 37°C overnight. Replicate nitrocellulose filters (Schleich & Schuell, NH) were made in duplicate from the overnight dishes, treated sequentially with 0.5M NaOH/1.5M NaCl, 1.5M NaCl/1.0M Tris-HCl (pH 8.0) and 2X SSC, and then baked at 80°C for 2 hours in a vacuum oven for DNA immobilization on the filters. Deoxynucleotide probes were end-labelled with T_4 polynucleotide kinase and γ-^{32}P-ATP (6,000 Ci/mmol., Du Pont NEN Research Products, MA). After 6 hour prehybridization, probe hybridization was carried out in a solution containing 40% formamide, 5X SSC, 5X Denhardt's solution, 0.2% SDS, 10mM EDTA, 25mM NaPi (pH 7.0) and 100μg/ml denatured herring sperm DNA, with the presence of $1X10^6$ cpm/ml of ^{32}P-labelled probe (specific activity $> 5X10^7$ cpm/μg) at 45°C overnight in a shaking waterbath (Precision, IL). Nitrocellulose filters were then washed in 0.5X SSC/0.1% SDS for 15 minutes, twice at room temperature and

twice at 42^{0}C. Filters were air-dried and subjected to autoradiography at -80^{0}C overnight (Sambrook *et al.*, 1989). Clones with positive signals on both duplicate filters were selected and processed for further rounds of hybridization and purification until all the plaques on the petri dishes gave positive signals in autoradiography. The final positive clones were subcloned into the *Not*I site of plasmid pBluescriptII SK+ (Stratagene, CA). Restriction enzyme mapping was established with restriction endonucleases *Apa*I, *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I and *Sal*I, both on the lambdaphage clones and plasmid clones using standard method of double digestion and Southern blotting analyses (Sambrook *et al.*, 1989). Restriction enzymes and modifying enzymes were purchased from New England Biolabs, MA, United States Biochemical Corporation, OH and Boehringer Mannhein Biochemicals, IN.

Polymerase chain reaction(PCR): Genomic DNA was extracted from both 129SV and C57/Black mouse brain, Sprague-Dawley rat brain and human placenta using standard phenol/chloroform method (Sambrook *et al.*, 1989). To detect the novel intron for verification, flanking primers D3042 and D3043N (Table 10-1) were designed based on rat sequence (Giros *et al.*, 1991), and the genomic DNA prepared from mouse and rat brain were used as templates (with clone SV_5X_{11} as control). Corresponding primers hD3040 and hD3041 (Table 10-1) were designed based on human D_3 cDNA sequence (Giros *et al.*, 1990) for detecting the analogous intron in the human D_3 gene. Polymerase chain reaction was performed

in a thermocycler at 10mM Tris-HCl (pH 8.8), 50mM KCl, 1.5mM $MgCl_2$, 0.001% (w/v) gelatin, 200μM each dNTP, 1μM each primer, 2 units of *Taq* DNA polymerase and 1 unit of perfect match DNA polymerase enhancer (Stratagene, CA) in a 50μl reaction volume. After the first denaturation at 94°C for 90 seconds, the reaction was set for 32 cycles of 94°C for 90 seconds, 60°C for 30 seconds and 75°C for 60 seconds, followed by a final extension at 75°C for 5 minutes. The products from polymerase chain reaction were resolved in 6% polyacrylamide gels followed by Southern blotting using nylon membranes in 0.5M NaOH/1.5M NaCl. After DNA immobilization and prehybridization, the membranes were hybridized with end-labelled marker D3046 (Table 10-1) overnight and then subjected to autoradiography.

Nucleotide sequence analysis: Sanger's dideoxynucleotide chain termination reaction method (Sanger *et al.*, 1977) was employed for sequence analysis on genomic and plasmid clones in both directions with appropriate primers. Firstly, the appropriate DNA bands from polymerase chain reaction products were excised from agarose or polyacrylamide gels after electrophoresis. The DNAs were then extracted from the gel slices and subcloned in plasmid pBluescript SK+ (Stratagene, CA) at *Eco*RV site. DNA minipreparations used for sequence analysis were prepared after plasmid transformation of DH5α™ competent cells (Bethesda Research Laboratory, MA). Sequencing reactions were carried out by using Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical

Corporation, OH) and α-^{35}S-dATP (1000Ci/mmol., Du Pont NEN Research Products, MA). The sequencing reaction products were resolved in 6% polyacrylamide wedged gels in the presence of 8M urea in 1X TBE (Sambrook *et al.*, 1989). The gels were then fixed with 10% (v/v) acetic acid and 10% (v/v) methanol for 30 minutes, dried in a gel dryer at 80^{0}C for 30 minutes under a vacuum hook and exposed to 35X43cm X-ray films (Kodak, NY) at room temperature overnight. Comparison was made both between the mouse sequence and rat sequence (Giros *et al.*, 1991), and between mouse cDNA and genomic sequences.

10C. Results

Six dopamine D_3 receptor clones were isolated from a mouse genomic library 129SV. Clones SV_1D_{11}, SV_2F_{121} and SV_3G_{111} were found to contain exons 1, 2 and 3, respectively. Clone SV_5X_{11} contained exons 4-7, and both clones SV_5U_{11} and SV_5W_{11} contained exons 6 and 7 (Figure 10-1). Sequence analysis showed that these clones contained the entire coding nucleotide sequence of the mouse dopamine D_3 receptor. All the exons with their boundaries to introns were sequenced and aligned to establish the genomic organization. The coding region of the mouse D_3 receptor gene was found to span at least 43 kilobases which is longer than the corresponding region of the rat D_3 receptor gene (Giros *et al.*, 1991) due to the longer length of mouse D_3 introns 1, 2, 3 and 5 (Table 10-2). The

3' part (20.5 kilobases) of the mouse D_3 receptor gene was mapped with various restriction endonucleases on clones SV_3G_{111}, SV_5U_{11}, SV_5W_{11} and SV_5X_{11} (Figure 10-2). The coding nucleotides of the mouse D_3 receptor have a 94.4% identity (1263 out of 1338) with that of the rat D_3 receptor while their homology at the amino acid level is 96.9% (432 out of 446, Figure 10-3). In agreement with a previous report on the sequence of the rat D_3 receptor gene (Giros *et al.*, 1991), we detected a splicing consensus sequence at -34 of the first ATG codon in the mouse D_3 gene, which suggests that an additional exon might exist further upstream. We also detected a mouse genomic codon CGA for arginine 226 while an AGA was detected by Fishburn *et al.* (1993) for this amino acid in a mouse cDNA sequence. Since the corresponding arginine in rat is also encoded by a CGA codon (Giros *et al.*, 1991), the AGA codon in the mouse cDNA might be due to allelic variation or a replication or sequencing error. In addition, the mouse D_3 receptor gene contained a cytosine at coding nucleotide position 415 (Cytosine 543 in Figure 10-3), same to that of the rat mRNA transcript D_3R-del reported by Snyder *et al.* (1991). This cytosine allowed D_3R-del mRNA to predict a 109 amino acid peptide which is 9 amino acids longer than the peptide encoded by another D_3 mRNA variant D_3(TM3-del) reported by Giros *et al.* (1991). The latter contained a guanine at the same position forming an earlier stop codon, that resulted in a peptide of 100 amino acids in length.

Analyses of the intron-exon organization and nucleotide sequence of

genomic clone SV_5X_{11} revealed an unexpected insert sequence of approximately 1 kilobase in length located between nucleotides 723 and 724 (encoding amino acids 241 and 242, respectively) of the full length D_3 mRNA. The 5' end of this insert contains an intron donor splicing sequence GA and the 3' end contains an intron acceptor sequence AG (Figure 10-3). Reading of this insert sequence in the same frame as the preceeding exon sequence revealed a stop codon TAG at position 66 after nucleotide 723. These observations suggested that this 1 kilobase insert found in clone SV_5X_{11} is a novel intron never reported before in the D_3 receptor gene. To verify the presence of this intron in the mouse genome, and to examine whether a similar intron would also be present in rat genome, we performed polymerase chain reaction analysis on genomic DNA prepared from both species using primers D3042 and D3043N which flank position 723/724. The products from polymerase chain reactions were then identified by Southern blot hybridization with the oligonucleotide probe D3046 as an internal marker derived from exon sequence located between these two primers (Table 10-1). It can be seen in Figure 10-5 that a DNA fragment of about 1.2 kilobases was detected in both rat and mouse genomes. Analysis of the subcloned DNA fragments from polymerase chain reactions indicated that the mouse genome contains a 1 kilobase intron identical in both size and sequence to the novel intron found in clone SV_5X_{11}. The rat genome contains a 1 kilobase intron highly homologous (about 91% similarity from the sequenced nucleotides) to the mouse novel intron, both having the same donor and acceptor consensus sequences and having the same stop codon at the same

position (data partially shown in Figures 10-3 and 10-7). It is thus confirmed that the novel intron is present in both rat and mouse genomes. This intron (termed intron 4) splits former exon 4 of the rat D_3 receptor gene into exons 4 and 5 containing 197 and 78 nucleotides, respectively. Former termed exons 5 and 6 are therefore designated here as exons 6 and 7, respectively (Figure 10-4). It is concluded that both D_2 and D_3 receptor genes have the same number of introns (six introns) in the coding region. The location and size of all introns of both D_2 and D_3 genes are compared in rat and mouse (Table 10-2). We notice that the location of the novel D_3 intron (intron 4) and its D_2 counterpart (intron 5) is similar in both D_2 and D_3 receptor genes, and their sizes are comparable. To eliminate the possibility of polymerase chain reaction amplification of the D_2 intron analogue, the primers D3042 and D3043N used in the polymerase chain reaction detection were so designed that they shared 95% and 97% homology respectively with the D_3 gene and only shared 55% and 52% homology with the D_2 gene. Consequent sequence analysis excluded the possibility of the D_2 gene amplification and confirmed that only the D_3 introns were detected.

To examine whether an equivalant intron would be present in human genome, we performed polymerase chain reaction analysis on human genomic DNA using primers hD3040 and hD3041 derived from the corresponding region of the human D_3 cDNA sequence (Giros *et al.*, 1990). The DNA products from the polymerase chain reactions gave two major DNA fragments on polyacrylamide

gels which were detected by Southern hybridization with probe D3046 as an internal marker (Figure 10-6). Sequence analysis on the subcloned plasmids indicated the lower band (106 basepairs + primers) contained a continuous coding sequence of 106 nucleotides derived from the human D_3 cDNA (Giros *et al.*, 1990). The upper DNA fragment (300 basepairs + primers) contained the expected 97 nucleotides of exon sequence followed by an insert of 203 nucleotides which started with a putative splicing donor sequence GT. This insert is situated at the same location as the newly defined murine introns (starting after nucleotide 723) and shares high (about 70%) nucleotide sequence homology with the 5' portion of these novel murine introns (data partially shown in Figure 10-7). Reading of the nucleotide sequence of this insert in the same frame as the D_3 mRNA revealed a stop codon TGA which is 48 nucleotides from the 3'-end of the proceeding exon. We suggest that this 203 basepair insert represents the starting segment of a human D_3 intron analogous to intron 4 newly defined in the rodent D_3 genes. We failed to detect the 3'-end of the putative intron in the human gene probably due to a random priming in the polymerase chain reactions. These data indicate that the human genome may contain two dopamine D_3 receptor genes, one with and another without this intron.

10D. Discussion

The dopamine D_3 receptor has been suggested to be a D_2-like receptor

(Sibley, 1991; Sibley and Monsma, 1992), and to share a common phylogenic genomic origin with the D_2 receptor (Giros *et al.*, 1991; O'Dowd, 1993) although they are located on different chromosomes (Grandy *et al.*, 1989a; Le Coniat *et al.*, 1991). The gene encoding the D_2 receptor has been well-characterized (O'Malley *et al.*, 1990; Mack *et al.*, 1991) except for the promoter region where controversies still exist (Minowa *et al.*, 1992b; Buck *et al.*, 1992; Minowa *et al.*, 1993b; Valdenaire *et al.*, 1994). The coding nucleotides of the D_2 receptor are spreaded on seven exons interspersed by six introns, and another intron is present in the 5' non-coding region of the gene. In contrast, the coding sequence of the D_3 receptor has been reported to be encoded by six exons because the D_3 nucleotide sequence corresponding to exons 5 and 6 in the D_2 gene was reported to be encoded by a single exon (exon 4) in the D_3 gene (Figure 9-6). Besides, it has not been confirmed yet if a corresponding intron is also present in the 5' non-coding region of the D_3 gene (Giros *et al.*, 1991). Here we report that both the rat and mouse D_3 genes contain an intron of 1 kilobase in length termed intron 4 which corresponds to intron 5 of the D_2 gene. This D_3 intron splits former-termed exon 4 into two exons, exon 4 and exon 5, containing 197 and 78 nucleotides, respectively. The discovery of the novel intron 4 in the D_3 receptor gene shows that the intron-exon organization of this gene is similar to that of the D_2 receptor gene. Their six corresponding introns are located at either the same or very similar nucleotide positions. We notice that the novel intron in the D_3 gene is actually the only one who shares the same location and similar size with its D_2 counterpart

(Table 10-2). In view of their high similarity in both sequences and genomic organization, we suggest the divergence of the D_2 and D_3 receptor genes may result from a relatively recent evolutionary event.

The D_2 coding exons in mouse, rat and human are clustered in a stretch of 11-13 kilobases in the genome (Mack *et al.*, 1991; O'Dowd, 1993). The rat D_3 coding region was reported to stretch for more than 18.5 kilobases, and every intron (except for the last one) was bigger than its D_2 counterpart (Giros *et al.*, 1991). In this study, we propose that the mouse D_3 receptor gene is even larger since most of the introns are significantly larger in mouse than in rat. The entire coding region of the mouse D_3 receptor gene spans at least 43 kilobases.

It is noteworthy that the deciphering of intron-exon organization of the human D_3 receptor gene has not been completely achieved yet. Our preliminary data from the polymerase chain reaction analysis on the human genome suggest the existence of an intron analogous to the one newly defined in the rodent D_3 receptor genes, implicating the species convergence of the D_3 receptor genes. On the other hand, our data further suggest that the human D_3 receptor may arise from at least two genomic loci, one containing and another lacking this novel intron, setting an example for species divergence for the dopamine D_3 receptor genes. The human D_3 locus which lacks this intron may represent a retropseudogene.

Table 10-1. Deoxynucleotide probes used in library screening and polymerase chain reaction.

PROBE	SIZE	DIRECTION	POSITION*(exon)	SEQUENCE
D3001	36mer	antisense	100-65(1)	5'-GGAGTTGAGGTGGGTGCTTATCTGGCTCAGAGGTGC-3'
D3002	40mer	sense	367-406(2)	5'-ACAGGTGGAGTCTGGAATTTCAGCCGCATTTGCTGTGACG-3'
D3003	41mer	antisense	640-600(3)	5'-CCAAAGAGGAGAGGGCAGGACACAGCAAAAGCCAGCACCCA-3'
D3005	41mer	antisense	1347-1307(5)	5'-TTCTTCTCTCGAAGTGGTACTCCCCGAGGCTGCAGGGGCCC-3'
D3042	20mer	sense	800-819(4)	5'-GTCCTGAGGCAAAGGCAAAG-3'
D3043N	23mer	antisense	1001-979(5)	5'-CATTTGTCCTGTGGCATCTGACN-3'
D3046	22mer	sense	825-846(4)	5'-GGATCCTCACTCGACAGAACAG-3'
D3051	22mer	antisense	1269-1248(6)	5'-TTTCGAACCTCTAAGCTGAGCT-3'
hD3040	21mer	sense	766-786#	5'-GGTGACTGTCCTTGTCTATGC-3'
hD3041	21mer	antisense	953-933#	5'-CTCCAGATGTGCCGGGTCAGG-3'

* Position is indicated as nucleotide numbers based on the D_3 sequence map (Figure 10-3);
\# Sequence is specific to human D_3 mRNA (Giros *et al.*, 1990).

Table 10-2. Comparison of location and size of introns in the dopamine D_2 and D_3 receptor genes.

The mouse D_3 data are compared with the published D_2 and D_3 data. The asterisk denotes the newly defined 1 kb intron in the rat and mouse D_3 genes. Deoxynucleotide sequence numbers are aligned on cDNA relative to the first ATG codon. Intron **locations** are indicated as the number of the nucleotide immediately 5' of the intron donor site. Intron **size** is given in parentheses () with kilobase as the unit. The presence of an additional intron at -35 in the D_3 gene is unclear. Note that the newly characterized D_3 intron and its D_2 equivalent intron are the only one who has the same location and similar size in the D_2 and D_3 genes.

D_2		D_3	
rat	mouse	rat	mouse
-32 (>25)	-32 (>18)	-35?	-35?
285 (~3.2)	285 (4.1)	270 (>5)	270 (>15)
395 (~1.5)	395 (0.8)	383 (>4)	383 (>11)
532 (~1.7)	532 (1.3)	526 (>4)	526 (9.5)
723 (~0.9)	723 (0.7)	723 (1.0)*	723 (1.0)*
810 (~1.4)	810 (1.8)	801 (2.6)	801 (3.7)
1141 (~2.3)	1141 (1.7)	1144 (1.1)	1144 (1.1)

Figure 10-1. Schematic intron-exon organization of the mouse dopamine D_3 receptor gene. (page 49)

The gene length is shown in scale except for the gaps (//) where the estimated size of intron is given in Figure 10-3. The exons are boxed and numbered, and the introns are shown as lines. The six genomic clones (see text) are aligned underneath. The entire gene spans over 43 kilobases.

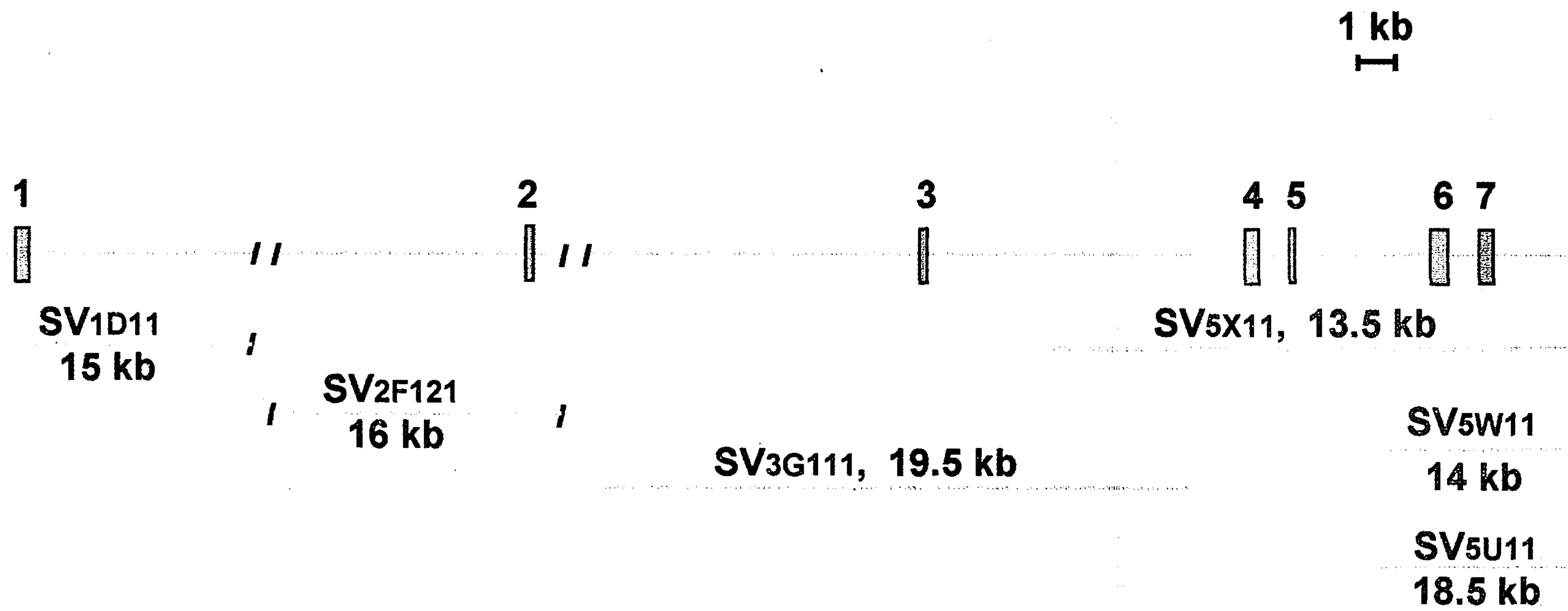
1 kb
1
2
3
4
5
6
7
SV1D11
15 kb
SV2F121
16 kb
SV3G111, 19.5 kb
SV5X11, 13.5 kb
SV5W11
14 kb
SV5U11
18.5 kb

Figure 10-2. Restriction endonuclease map of the mouse dopamine D_3 receptor gene. (page 51)

An *Eco*R I fragment of 20.5 kilobases of the gene which covers exons 3-7 was mapped. Double digestion and Southern blotting techniques were employed for the mapping. The exons are boxed and numbered, and the introns are shown as lines. Restriction enzyme sites: A(*Apa* I), B(*Bam*H I), H(*Hin*d III), K(*Kpn* I), P(*Pst* I), R(*Eco*R I), S(*Sal* I) and V(*Eco*R V).

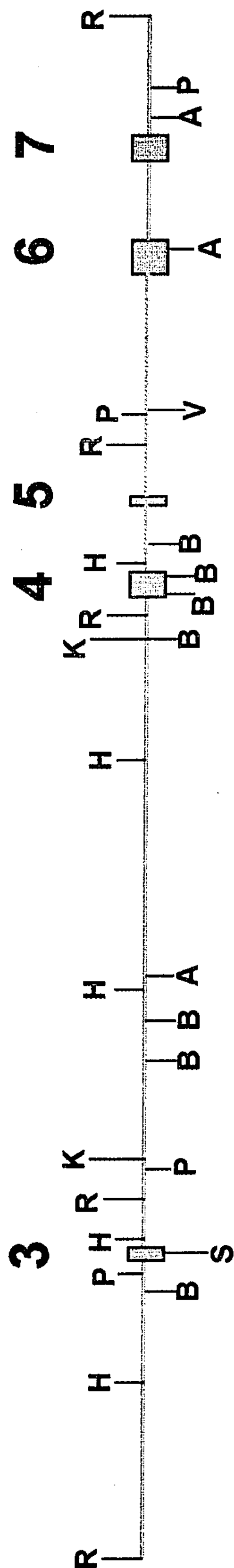
1 kb
3
4
5
6
7

Figure 10-3. Sequence map of the mouse dopamine D_3 receptor gene. (pages 53 and 54)

The intronic sequences are given in lower case letters. The translated amino acids are given under the nucleotide codons. The different nucleotides and amino acids of the **rat** D_3 receptor from the mouse D_3 receptor are superscripted on the mouse sequences except for the dotted parts (----) where no rat data are available. The predicted *trans*-membrane domains TM1-7 are overlined. An asterisk indicates the internal acceptor site where the alternative splicing occurs. The size of each of introns 3-6 is obtained from restriction mapping, and the size of each of introns 1 and 2 is estimated from the size of the genomic clones.

```
                                                                                               Thr     Leu
    c    c                  G               C          G                                         C       C
cccatattgttgtctgtcttttcctag AAACATTTTGGAGTCGGGTTCCTCTGTCTGGGCC  ATG GCA CCT CTG AGC CAG ATA AGC AGC CAC ATC AAC     97
                                                                Met Ala Pro Leu Ser Gln Ile Ser Ser His Ile Asn     12

          C                           C                       G   C         __________________________T_______
TCC ACC TGT GGG GCA GAA AAC TCC ACT GGT GTC AAC CGG GCC CGT CCA CAT GCC TAC TAC GCC CTG TCC TAC TGT GCA CTC ATC    181
Ser Thr Cys Gly Ala Glu Asn Ser Thr Gly Val Asn Arg Ala Arg Pro His Ala Tyr Tyr Ala Leu Ser Tyr Cys Ala Leu Ile     40

__A_______(TM 1)______________C_______________T___________                T       G
CTG GCC ATC ATC TTT GGC AAC GGT CTG GTA TGT GCA GCT GTG CTG AGG GAG CGA GCC CTA CAG ACC ACC ACC AAC TAC CTA GTG    265
Leu Ala Ile Ile Phe Gly Asn Gly Leu Val Cys Ala Ala Val Leu Arg Glu Arg Ala Leu Gln Thr Thr Thr Asn Tyr Leu Val     68

______________________C___________A___________G_______(TM 2)_________________________________          cccg
GTG AGC CTG GCT GTG GCA GAC CTG CTG GTG GCC ACT TTG GTG ATG CCG TGG GTG GTG TAC TTG GAG  gtaagtgggctttatg......    347
Val Ser Leu Ala Val Ala Asp Leu Leu Val Ala Thr Leu Val Met Pro Trp Val Val Tyr Leu Glu                             90

                       ctgt t ctctg     _______                                             __C_______________
..intron 1 (> 15 kb)...tgtctctactacccag GTG ACA GGT GGA GTC TGG AAT TTC AGC CGC ATT TGC TGT GAT GTT TTT GTC ACC    417
                                        Val Thr Gly Gly Val Try Asn Phe Ser Arg Ile Cys Cys Asp Val Phe Val Thr    108

______________________(TM 3)_____________________________________________           ga  acaac a at gg
CTG GAT GTC ATG ATG TGT ACA GCC AGC ATC CTG AAC CTC TGT GCC ATC AGC ATA GAC AG  gtagggctgggagttctca ...........    495
Leu Asp Val Met Met Cys Thr Ala Ser Ile Leu Asn Leu Cys Ala Ile Ser Ile Asp                                        127
                                                                                Glu
                     ----------------             G                             G     C
.intron 2 (> 11 kb)..ccttgctctttcacag G TAC ACA GCA GTG GTC ATG CCA GTT CAC TAT CAG CAT GGC ACC GGG CAG AGC TCC    566
                                    Arg Tyr Thr Ala Val Val Met Pro Val His Tyr Gln His Gly Thr Gly Gln Ser Ser    146

    A       ______A___________C___A_______________________T___(TM 4)_________________________________________
TGT CGA CGT GTG GCG CTC ATG ATT ACG GCT GTG TGG GTG CTG GCC TTT GCT GTG TCC TGC CCT CTC CTC TTT GGT TTC AAC ACA    650
Cys Arg Arg Val Ala Leu Met Ile Thr Ala Val Trp Val Leu Ala Phe Ala Val Ser Cys Pro Leu Leu Phe Gly Phe Asn Thr    174

      ----------------                              t                                               ___________
ACA G gttaaaataactattg..intron 3 (9.5 kb)...ccttctccaaccacag GG GAT CCC AGC ATC TGC TCC ATC TCC AAC CCT GAT TTT    724
Thr                                                         Gly Asp Pro Ser Ile Cys Ser Ile Ser Asn Pro Asp Phe    188
                                                                                                Ile
__________________A___(TM 5)__________C___________C_______________________________                A
GTC ATT TAC TCT TCG GTG GTG TCC TTC TAT GTT CCC TTT GGG GTG ACT GTC CTG GTC TAT GCC AGG ATC TAC ATG GTC CTG AGG    808
Val Ile Tyr Ser Ser Val Val Ser Phe Tyr Val Pro Phe Gly Val Thr Val Leu Val Tyr Ala Arg Ile Tyr Met Val Leu Arg    216
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(To be continued on next page)

(Continued from last page)

```
        Gln
         A                                                 C       T                   T                a     t
CAA AGG CGA AGA AAA CGG ATC CTC ACT CGA CAG AAC AGC CAG TGT ATC AGC ATC AGA CCT GGC TTC CCT CAG CAG gtatgcacccg   894
Gln Arg Arg Arg Lys Arg Ile Leu Thr Arg Gln Asn Ser Gln Cys Ile Ser Ile Arg Pro Gly Phe Pro Gln Gln               241

                               ct  t c t    ac                       A
gcaggagct....intron 4 (1 kb)...tctccttcaacctttcccag TCT TCC TGT CTG CGG CTG CAT CCC ATT CGG CAG TTT TCA ATA AGG   968
                                                    Ser Ser Cys Leu Arg Leu His Pro Ile Arg Gln Phe Ser Ile Arg   256

                              A                                                   c   c t
GCC AGG TTT CTG TCA GAT GCC ACG GGA CAA ATG gtaagtgggtgt...intron 5 (3.7 kb)...tttattgtcttccctttcag GAG CAC ATA  1042
Ala Arg Phe Leu Ser Asp Ala Thr Gly Gln Met                                                          Glu His Ile   270

            Gln                                                            Leu                             Gly
             A                                 C   T           C   G    *     C    A       T                 G  G
GAA GAC AAA CCA TAT CCC CAG AAA TGC CAG GAC CCT CTC TTG TCA CAT CTA CAG CCC CTC TCT CCT GGC CAG ACA CAT GGA GAG  1126
Glu Asp Lys Pro Tyr Pro Gln Lys Cys Gln Asp Pro Leu Leu Ser His Leu Gln Pro Pro Ser Pro Gly Gln Thr His Gly Glu   298

                                                                Ser Leu             Ala             Pro
      G                                                A   C      G    G       C      C               CC       A
CTG AAA CGC TAC TAC AGC ATC TGC CAA GAC ACT GCC TTG AGG CAT CCA AAC TTC GAA GGA GGG GGA GGG ATG AGC CAA GTG GAG  1210
Leu Lys Arg Tyr Tyr Ser Ile Cys Gln Asp Thr Ala Leu Arg His Pro Asn Phe Glu Gly Gly Gly Gly Met Ser Gln Val Glu   326

                      G                                                                    C                   G
AGG ACT CGG AAC TCC TTA AGC CCC ACC ATG GCA CCC AAG CTC AGC TTA GAG GTT CGA AAA CTC AGC AAT GGC AGG TTA TCC ACA  1294
Arg Thr Arg Asn Ser Leu Ser Pro Thr Met Ala Pro Lys Leu Ser Leu Glu Val Arg Lys Leu Ser Asn Gly Arg Leu Ser Thr   354

        Arg
         G                 G                                                                               T
TCC CTG AAG CTG GGG CCC CTA CAG CCT CGG GGA GTA CCA CTT CGA GAG AAG AAG GCC ACC CAG ATG GTG GTC ATT GTG CTC G g  1377
Ser Leu Lys Leu Gly Pro Leu Gln Pro Arg Gly Val Pro Leu Arg Glu Lys Lys Ala Thr Gln Met Val Val Ile Val Leu       381

      c        cg                                        A                   C   (TM 6)           C
taagtctgagttgctag..intron 6 (1.1 kb)..ctttctttgcaacccag  GG GCC TTC ATT GTC TGT TGG CTG CCC TTC TTC TTG ACT CAC  1452
                                                        Gly Ala Phe Ile Val Cys Trp Leu Pro Phe Phe Leu Thr His   395

                          A                                                A   G       A       T       (TM 7)
GTT CTT AAT ACC CAC TGT CAG GCA TGC CAC GTG TCC CCA GAG CTT TAC AGA GCC ACG ACA TGG CTT GGC TAC GTG AAC AGT GCC  1536
Val Leu Asn Thr His Cys Gln Ala Cys His Val Ser Pro Glu Leu Tyr Arg Ala Thr Thr Trp Leu Gly Tyr Val Asn Ser Ala   423

                                        Val
      T               T               T G G                                C   G                            C
CTG AAC CCT GTG ATC TAC ACC ACC TTC AAC ATA GAG TTC CGC AAA GCC TTC CTC AAG ATT CTA TCC TGC TGAAGGAGGAGAAGAGACA  1624
Leu Asn Pro Val Ile Tyr Thr Thr Phe Asn Ile Glu Phe Arg Lys Ala Phe Leu Lys Ile Leu Ser Cys STOP                  446
```

Figure 10-4. Schematic showing the newly-defined intron in the dopamine D_3 receptor gene. (page 56)

The former-termed and newly-defined murine D_3 receptor genes are compared. The exons are boxed and numbered, and the introns are shown as dotted lines. The length is not in scale. Note the 1 kilobase newly-defined intron (intron 4) splits former exon 4 in rat into exons 4 and 5. Former exons 5 and 6 therefore become exons 6 and 7, respectively.

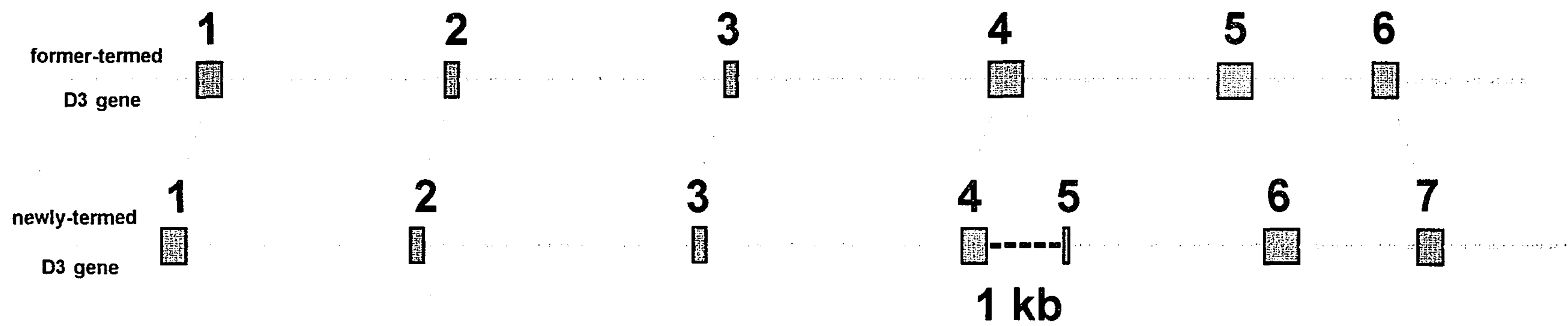

former-termed
D3 gene
1
2
3
4
5
6
newly-termed
D3 gene
1
2
3
4
5
6
7
1 kb

Figure 10-5. Polymerase chain reaction products showing the novel intron in the rat and mouse dopamine D_3 receptor genes. (page 58)

The left panel shows a 0.7% agarose gel which visualizes 1.2 kilobase DNA bands from PCR products of rat (**R**), mouse (**M**) genomic DNA with clone SV_5X_{11} (**X**) used as control templates. Gel was stained with 0.5 µg/ml ethidium bromide (**mk**: DNA molecular weight marker). The right panel shows the autoradiography of Southern blotting from the left panel's gel. End-labelled oligonucleotide D3046, an internal marker, stains the 1.2 kilobase bands. These 1.2 kilobase DNA bands were further analysed and found to contain a 1 kilobase intron between exon sequences (Figures 10-3 and 10-7).

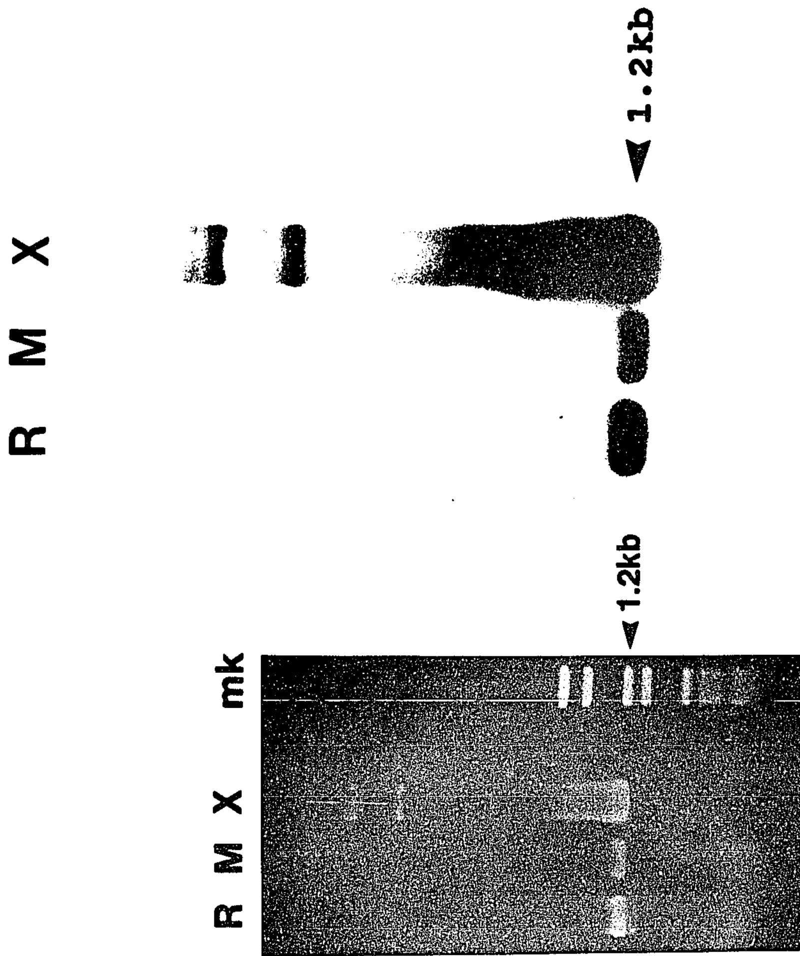
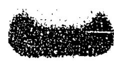

R M X
1.2kb
R M X mk
1.2kb

Figure 10-6. Southern blot of polymerase chain reaction (PCR) products from human genomic DNA. (page 60)

PCR was performed using human genomic DNA (**H**) as templates and oligonucleotides hD3040 and hD3041 as primers (Table 10-1). Two major DNA bands were obtained from electrophoresis of the PCR products on a 6% polyacrylamide gel. The approximately sizes of these two bands were 350 and 150 basepairs, respectively. Southern blotting analysis shows here that both DNA bands were hybridized with end-labelled probe D3046 (Table 10-1). For further sequence analysis and interpretation, see right-most panel of Figure 10-7 and the text.

H

394bp ➤



154bp ➤

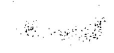
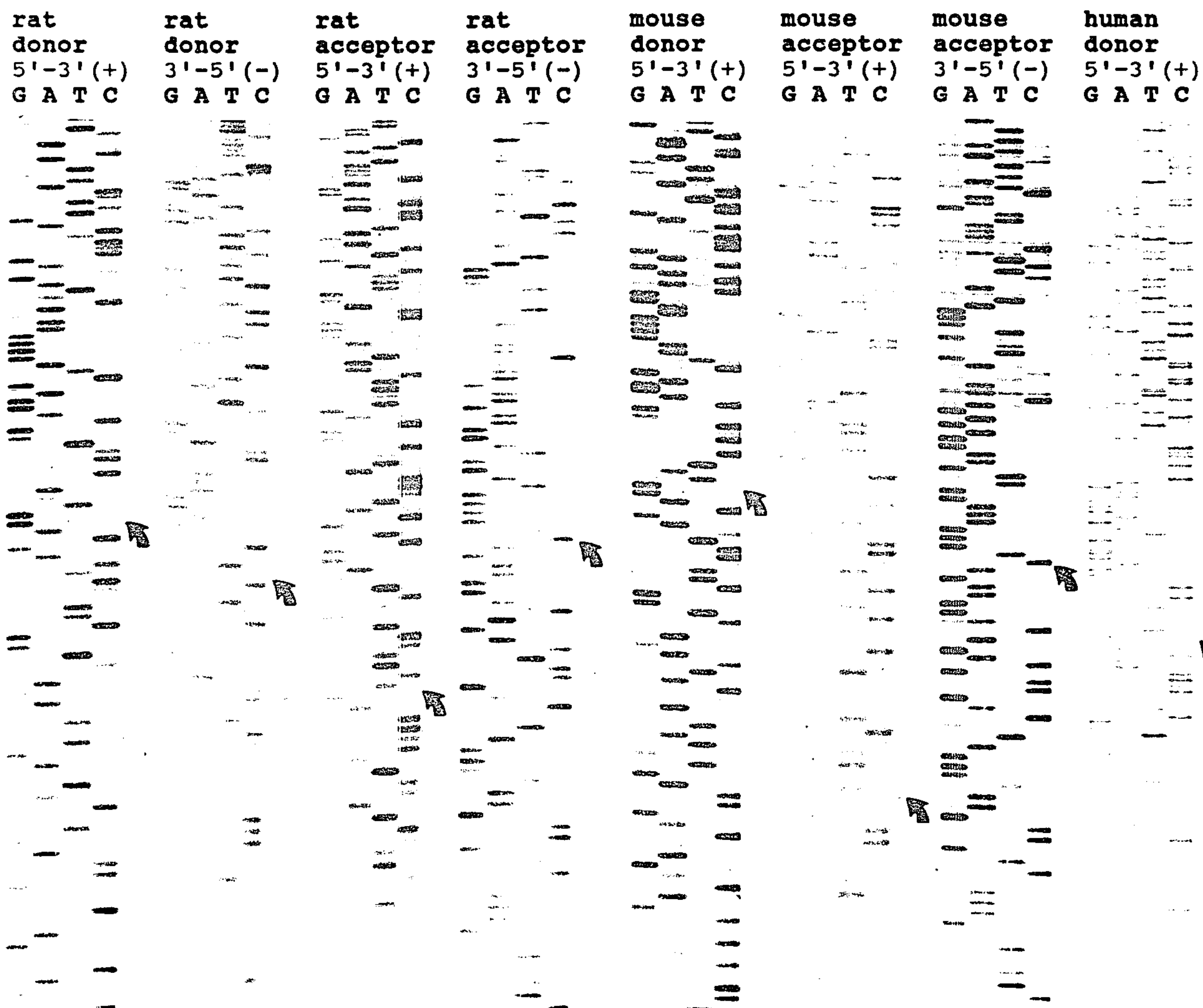


Figure 10-7. Sequencing gels showing the splicing sites of the novel intron found in the rat, mouse and human dopamine D_3 receptor genes. (page 62)

DNA bands from PCR products (see Figures 10-5 and 10-6) were subcloned in pBluescript SK+, and amplified and used as sequencing templates. The T_3 and T_7 promoter-specific primers and D_3 gene-specific primers were used to direct sequencing reactions in both directions on both DNA strands. Arrows indicate both the sequence reading directions and intron-exon boundaries. Splicing donor and acceptor sites being searched in the gels are as marked. (+) designates sequences on the forward DNA strands, and (-) designates the complementary DNA strands.


rat donor 5'-3'(+) G A T C
rat donor 3'-5'(-) G A T C
rat acceptor 5'-3'(+) G A T C
rat acceptor 3'-5'(-) G A T C
mouse donor 5'-3'(+) G A T C
mouse acceptor 5'-3'(+) G A T C
mouse acceptor 3'-5'(-) G A T C
human donor 5'-3'(+) G A T C

PART THREE: The finding and characterization of a short mRNA variant for the mouse dopamine D_3 receptor arising from post-transcriptional alternative mRNA splicing

11A. Introduction 64

11B. Materials and methods 68

11C. Results 70

11D. Discussion 71

Figure 11-1 75

Figure 11-2 77

Figure 11-3 79

11A. Introduction

Eukaryotic mRMA variants derive from alternative splicing during post-transcriptional processing of mRNA precursors. Splicing usualy takes place at intron-exon junctions, but it can also occur within exons. They all reflect functional divergence of the biological macromolecules, species and tissue specificities of proteins, or sometimes even the generation of new protein species.

The first member of the dopamine receptor family cloned was a rat D_2 genomic clone followed by a D_2 cDNA clone. The open reading frame of the coding sequence predicted that the rat D_2 receptor contained 415 amino acids (Bunzow *et al.*, 1988). Following this finding, many groups reported a second D_2 cDNA form encoding a D_2 receptor protein with a 29 amino acid insert in the putative third intracellular loop (Dal Toso *et al.*, 1989; Eidne *et al.*, 1989; Selbie *et al.*, 1989; Giros *et al.*, 1989; Grandy *et al.*, 1989; Chio *et al.*, 1990; O'Malley *et al.*, 1990; Robakis *et al.*, 1990; Martens *et al.*, 1991). Now it is clear that both D_2 isoforms derive from the same gene by alternative mRNA splicing of a small cassette exon encoding 29 amino acids of the putative third intracellular loop. Actually, it was the first time for a member of the G protein-coupled receptor superfamily to be found to use alternative mRNA splicing to generate receptor mRNA isoforms. Northern blot and *in situ* hybridization analyses revealed that both D_2 mRNA isoforms co-exist in all areas where the D_2 receptor is expressed.

Their relative proportions vary from one area to another and the short form seems to be generally less abundantly transcribed (Dal Toso *et al.*, 1989; Monsma *et al.*, 1989; Sibley *et al.*, 1992). Furthermore, Neve *et al.* (1991) reported that although dopamine-depleting lesions in the denervated neostriatum increased the density of the D_2 receptors by 29%, the ratio of D_{2Long} over D_{2Short} was not altered.

The variation of the relative abundance of the two D_2 receptor mRNA isoforms reflects their different tissue distribution. For instance, D_{2Long} mRNA was found to be expressed in the pituitary at a level six folds higher than D_{2Short} mRNA. The D_{2Long} was also more abundant in striatum; however, both were expressed almost equally in hippocampus, kidney and anterior cortex (Neve *et al.*, 1991). Since the two isoforms differ in a region shown to be important for G protein-coupling (Ross, 1989), it is likely that this alternative splicing is used to fine-tune receptor interactions with G_i and/or G_o proteins. In fact, Dal Toso *et al.* (1989) stated that the D_{2Short} form inhibited adenylate cyclase activity to a greater extent than did the D_{2Long} form, commensurate with the notion that the short form more effectively couples with a G_i protein. However, no functional differences in their pharmacological profiles were determined when using known specific D_2 receptor ligands (Giros *et al.*, 1989). It is still too early to exclude a functional variance between D_{2Long} and D_{2Short} because the differences created by a small peptide insertion in the third intracellular loop may be elusive by using conventional pharmacological approaches. A recent paper reported that D_{2Short} and D_{2Long} were

differentially regulated in response to dopamine exposure (Zhang *et al.*, 1994).

Searching for the dopamine D_3 mRNA variants yields a more complicated story. Giros *et al.* (1991) first reported two short D_3 receptor variants in rat, termed D_3(TM3-del) and D_3(O2-del), resulting from various patterns of alternative mRNA splicing as follows. D_3(TM3-del) was derived from a D_3 mRNA transcript which lacked a stretch of 113 nucleotides mainly corresponding to the putative third *trans*-membrane domain. This deletion resulted in a protein product of only 100 amino acids long due to a premature termination of translation caused by a reading frame shift. This 113 nucleotide deletion corresponding to exon 2 underlies a combinatorial exon (or called a "cassette" exon) splicing. On the other hand, D_3(O2-del) is a 428 amino acid protein arising from an mRNA transcript with a 54 nucleotide in-frame deletion. This deletion comprises the region encoding the 10 last amino acids of the second extracellular loop and the first 8 amino acids of the fifth *trans*-membrane domain. This 54 nucleotide deletion was believed to be a result of an mRNA splicing that occured within the fourth exon where an internal acceptor site was used by the splicing machinery. Although the structure of D_3(O2-del) protein may still be compatible with the occurence of seven *trans*-membrane domains, Chinese hamster overy clones stably expressing D_3(O2-del) mRNA failed to show any dopaminergic binding activity as assessed with various radioactive ligands (Giros *et al.*, 1991). Snyder *et al.* (1991) reported a short D_3 mRNA (D_3R-del) both in rat and human, which is identical to the mRNA variant encoding

D_3(TM3-del) except for a G to C substitution at nucleotide 415 of the full length D_3 mRNA, allowing D_3R-del to encode a 109 amino acid peptide which is 9 amino acids longer than D_3(TM3-del). Both D_3(TM3-del) and D_3R-del products are too short to display any dopamine receptor activity. Another short D_3 mRNA variant D3(TM4-del) missing exon 3 (143 nucleotides) was found in both human brain and lymphocyte mRNA. This 143 nucleotide deletion, encompassing mainly the fourth *trans*-membrane domain and the second intracellular loop, results in a 138 amino acid unfunctional peptide due to a premature termination of translation (Nagai, 1993). Another human short D_3 mRNA called D_{3nf} was recently reported (Schmauss *et al.*, 1993) to lack 98 nucleotides from the 3'-end of the nucleotide sequence encoding the third intracellular loop. D_{3nf} had a reading frame-shift and predicted a 342 amino acid peptide which was not functional. Despite the very low abundance of D_{3nf} mRNA, the expression of this mRNA was unaffected in the brain of patients with chronic schizophrenia, whereas the expression of the full length D_3 mRNA was found to be selectively lost in the motor and parietal cortices of these schizophrenic patients when compared to normal controls (Schmauss *et al.*, 1993). Besides, Pagliusi *et al.* (1993) reported a long form of rat D_3 receptor mRNA variant, $rD3^{in}$, which contained an insert of 84 nucleotides located between the sequences encoded by exons 1 and 2. This 84 nucleotide insertion was suggested to be transcribed from an extra exon contained within intron 1. This extra exon had never been reported before and it encoded 27 extra amino acids plus an in-frame TGA stop codon. It remains unclear whether these transcript

variants described above are formed randomly during the transcription of the functional dopamine D_3 receptor mRNA, and whether they serve regulatory or modulatory roles by controling the abundance of the active D_3 gene transcripts.

Recently, Fishburn *et al.* (1993) described a novel mRNA variant (called D_{3Short}) which predicted a protein exibiting binding activity for dopaminergic agonists and antagonists. This variant was revealed to lack 63 nucleotides in the putative third intracellular loop region which might result from using an internal acceptor splicing site. Our concurrent study agrees with their finding. We report here our data and discuss the nucleotide sequences encoding the third intracellular loop and their relation to the D_2 and D_3 mRNA splicing in different species.

11B. Materials and Methods

Reverse transcription and polymerase chain reaction: Total RNA was extracted from mouse and rat whole brain and human neocortex using guanidinium thiocyanate-cesium chloride method (Sambrook *et al.*, 1989). Reverse transcription reactions were performed using cDNA Cycle™ Kit (Invitrogen, CA) using AMV reverse transcriptase to produce first strand cDNA from mRNA. To detect the alternative splicing in the putative third intracellular loop coding region, oligonucleotides D3042 and D3051 (Table 10-1) which flank most part of this region were used as primers and the first strand cDNAs from reverse transcription

reaction of mouse and rat RNAs were used as templates in the polymerase chain reaction under the same condition as described in Materials and Methods, section 10B of this dissertation. Primers hD3040 and D3051 (Table 10-1) were used in polymerase chain reaction amplification of the corresponding region on the first strand cDNAs generated from the human mRNA by reverse transcription reactions.

Subcloning and sequence analysis: Products from polymerase chain reaction detections were electrophorised on agarose or polyacrylamide gels. DNA bands were transferred onto nylon membrane by either Southern blotting (for agarose gels) or electro-transferring (for polyacrylamide gels). The nylon membrane were hybridized with ^{32}P-lablled marker D3046 (Table 10-1) to confirm the right DNA bands were detected. For sequence analysis, each individual DNA band was carefully excised from the gels and the DNA were extracted and subcloned at the *Eco*RV site of plasmid pBluescript SK+ (Stratagene, CA). DNA preparations used for sequence analysis were prepared from the plasmid-transformed competent *E. coli* cells (DH5α™, Bethesda Research Laboratory, MA). The polymerase chain reaction products which show only a single DNA band in electrophoresis were also used for direct subcloning, with the DNA extraction step omitted. Alternatively, these products were direcetly used as templates to prepare single stranded DNA for sequencing in the presence of helper virus M13KO7 (Sambrook *et al.*, 1989). Sequencing reactions were carried out on both DNA chains using Sanger's dideoxy method as described in Materials and Methods, section 10B of this dissertation.

T_3 and T_7 promoter primers and reverse primers were used to direct the sequencing reactions.

11C. Results

Thus far, the cassette exon 6 has been reported to be the only alternatively spliced sequence in the dopamine D_2 receptor gene. To determine whether the same splicing event occurs with the analogous "cassette" exon 5 in the D_3 gene, reverse transcription-polymerase chain reaction experiments were performed using primers D3042 and D3051 which derive from exons 4 and 6 respectively and flank exon 5 (Table 10-1). Two bands were obtained from mouse RNA template in polyacrylamide gels (Figure 11-1), with the approximate sizes being 400 nucleotides and 340 nucleotides, respectively. The intensity of the lower band (340 nucleotides) was several folds lower than that of the upper band. Sequence analysis from subcloned plasmids showed that the upper band represents a continious coding sequence (full length D_3 mRNA) while the lower band represents a deleted form of the mouse D_3 receptor mRNA. Exon 5, however, is not alternatively spliced as expected from the D_2 situation, the real alternative splicing takes place within exon 6, resulting in the generaton of the D_{3Short} mRNA lacking a 21 amino acid-encoding sequence from the first part of exon 6 (Figure 11-2). This D_{3Short} mRNA encodes a 425 amino acid D_3 receptor form, 21 amino acids shorter in the third intracellular loop domain than the full length D_3 receptor form. Our results thus agree with the

data of Fishburn *et al.* (1993). Interestingly, this alternatively splicing event was only observed in mouse brain but not in rat brain as shown in Figure 11-1 where only one band of 400 basepairs (representing the full length D_3 mRNA) was obtained from rat sample under the same experimental conditions. Products from reverse transcription-polymerase chain reaction on human mRNA template consistently gave only one band (data not shown here), and the nucleotide sequence of this band agrees with the human dopamine D_3 receptor cDNA clone previously reported by Giros *et al.* (1990). These results hence suggested that the D_{3Short} mRNA might be specific to the mouse brain.

11D. Discussion

Our newly established intron-exon organization of the D_3 receptor gene prompted us to examine whether the splicing event at D_2 exon 6 could also occur at the corresponding exon 5 of the D_3 gene. Although we did find a D_{3Short} form, its "missing" part was a 63 nucleotide sequence (encoding 21 amino acids) derived from the 5'-region of exon 6 rather than the "cassette" exon 5. Hence, it is likely that a cryptic 3' splice site present in exon 6 is activated to become an alternative internal splicing acceptor. This site conforms in sequence to the criterion of the splicing acceptor consensus sequence "$(P_y)_nNP_y\underline{AG}$" (Mount, 1982; Green, 1986). It is noteworthy that, in either rat or mouse D_2 receptor gene, there is a similar consensus sequence only two bases away from the corresponding position in the D_3

gene, but none of this kind of splcing event was reported for the D_2 gene. On the other hand, a previously reported D_3 mRNA variant D_3(O2-del) was derived via the same splicing mechanism at another position with a very similar sequence (Giros *et al.*, 1991). However, the 428 amino acid protein expressed from D_3(O2-del) mRNA was reported to lack any binding activity (Giros *et al.*, 1991). Fishburn *et al.* (1993) first documented the D_{3Short} mRNA variant and further determined that its binding activity is similar to that of the full length D_3 receptor in an *in vitro* expression system. This D_3 mRNA variant has been the only one which predicted a short D_3 receptor protein with considerable binding activity for dopaminergic agents. Our results also show that, like D_2 isoforms, D_{3Long} is the predominant form over D_{3Short} in the mouse brain. It is interesting that D_{2Short} and D_{3Short} are derived via different post-transcriptional mechanisms involving different exons (Figures 11-3). The biological function of these two short isoforms remains to be further deciphered.

Interestingly, we detected the D_{3Short} mRNA isoform only from mouse brain but not from rat brain. As mentioned above, the intron-exon arrangement of the rat and mouse D_3 receptor genes is virtually identical, and the rat D_3 gene also contains a splicing acceptor consensus sequence at the same position of the mouse D_3 receptor gene where the splicing occurs. However, it is surprising that no corresponding short D_3 mRNA form was detected from rat brain (concluded from many trials). Other workers were also unable to detect the D_{3Short} mRNA from rat

(Dr. S. Fuchs, personal communications). We tried to analyse in more detail the nucleotide sequence of this part of the D_3 gene and found that the alternatively spliced nucleotides of the mouse D_3 gene share a 92.1% sequence homology (only 5 out of 63 bases are different) with the corresponding stretch of nucleotides of the rat D_3 receptor gene. These five different bases do not appear to constitute a big change in the polypyrimidine tract as observed from the conventional criterions for splicing (Green, 1986; Wiebauer *et al.*, 1988). Besides, the mouse intron 5 (immediately upstream of the spliced 63 nucleotides) shares high degree of sequence homology with its analogous intron in rat at both intron-exon boundaries (data partially shown in Figure 10-3) although the size of the intron is 3.7 kilobases in mouse versus 2.6 kilobases in rat (Table 10-2). It is conceivable that some important splicing features may exist in addition to the known criterions. This difference between rat and mouse D_3 nucleotides might provide a good model for exploring additional features of post-transcriptional splicing mechanisms. Mutational analysis in conjunction with *in vitro* and *in vivo* expression systems might be useful in this pursuit.

To examine whether the corresponding region in the human D_3 receptor gene is also alternatively spliced, we performed reverse transcription-polymerase chain reaction on human mRNA using primers specific to the human D_3 cDNA. Only one human D_3 receptor mRNA has been detected. In agreement with a previous report (Giros *et al.*, 1990), the human D_3 mRNA lacks a 138 nucleotide

sequence encoding 46 amino acids of the third intracellular loop when compared to the murine D_3 mRNA. These 138 "missing" nucleotides comprise the corresponding portion that is spliced out in the mouse D_{3Short} mRNA. So it is not possible for this human D_3 mRNA to further have this kind of splicing. On the other hand, the intron-exon organization of the human D_3 gene is not established. Our preliminary data (see Part II of this dissertation) suggest that the human D_3 receptor may have at least two genomic loci, one containing and another lacking an intron analogous to the newly defined intron 4 in the rat and mouse D_3 genes. The human D_3 gene missing this intron may represent a D_3 retropseudogene. We have no explanation at this point why the human D_3 gene and its mRNA show more species diversity than other dopamine receptor subtypes, and what the underlying physiological significance of this divergence might have.

Figure 11-1. Polyacrylamide gel showing RT-PCR products from rat and mouse brain mRNA. (page 76)

RT-PCR were performed using total rat (**R**) and mouse (**M**) brain RNA extracts as templates and oligonucleotides D3042 and D3051 as PCR primers (Table 10-1 and Figure 11-3, D_3). After electrophoresis on a 6% polyacrylamide gel and staining of the gel with 0.5 μg/ml ethidium bromide, two DNA bands were achieved from the mouse sample with the approximately sizes of 400 and 340 basepairs, respectively. But only a 400 basepair corresponding band was obtained from the rat sample under the same experimental conditions. In the mouse column, the intensity of the lower band is several folds lower than that of the upper band. **mk**: DNA molecular weight marker.

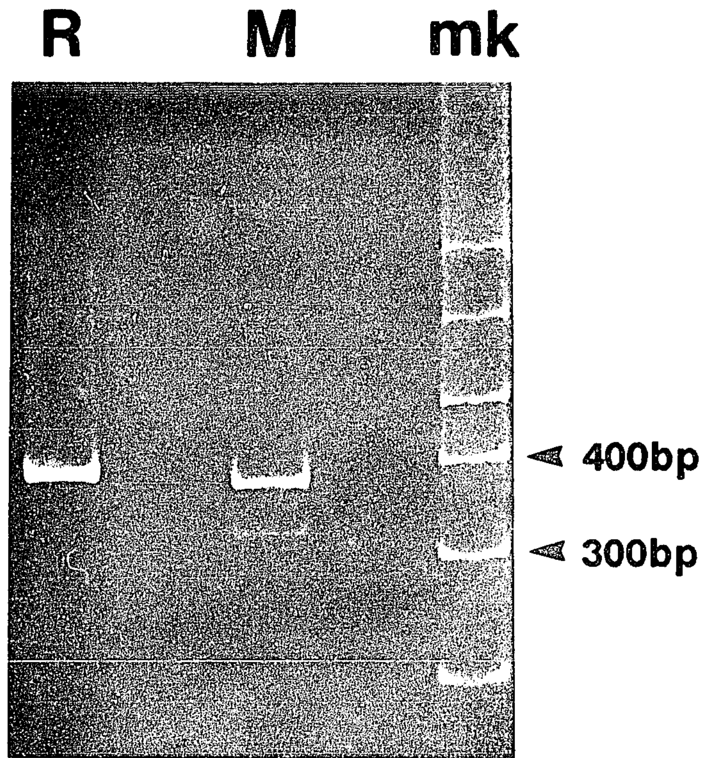

R
M
mk
400bp
300bp

Figure 11-2. Sequencing gel showing the alternatively splicing position of the D_{3Short} mRNA found in the mouse brain. (page 78)

DNA bands from RT-PCR products (Figure 11-1) were subcloned in pBluescript SK+, and amplified and used as sequencing templates. This picture shows the result of nucleotide sequencing on the 340 basepair band of the mouse sample (Figure 11-1), which represents the D_{3Short} mRNA isoform. An arrow indicates both the sequence reading direction and the position where alternative splicing occured. Note that 63 nucleotides of the full length D_3 mRNA were spliced out at this position (please refer to Figure 10-3).

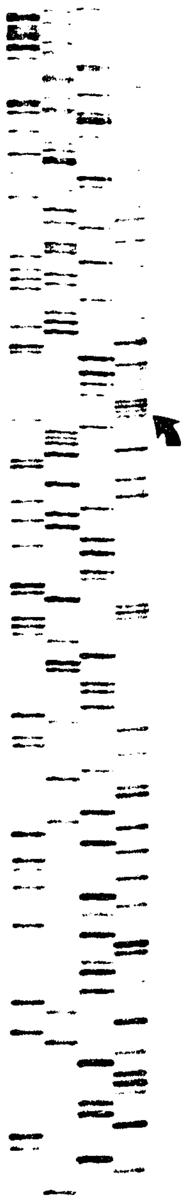
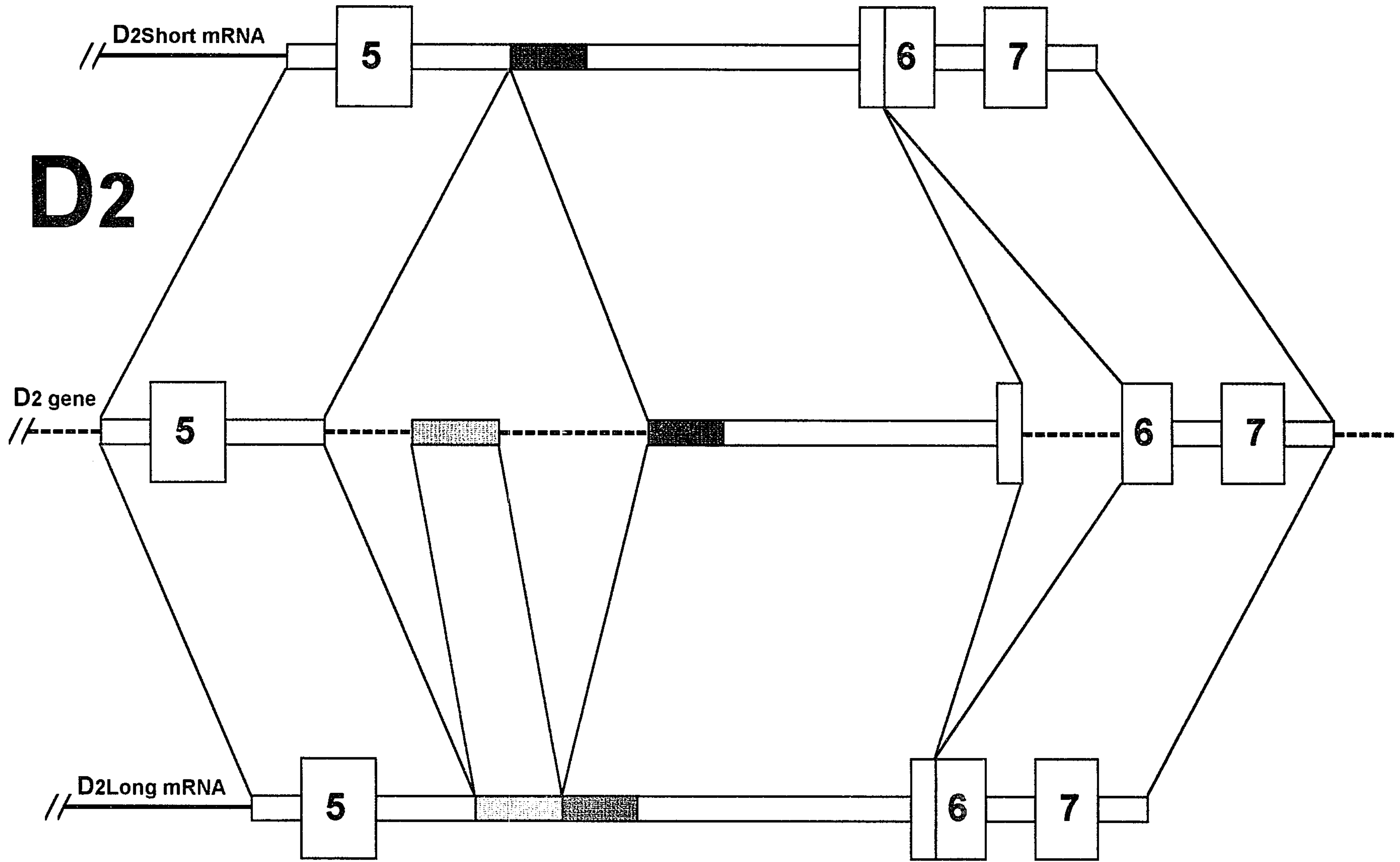
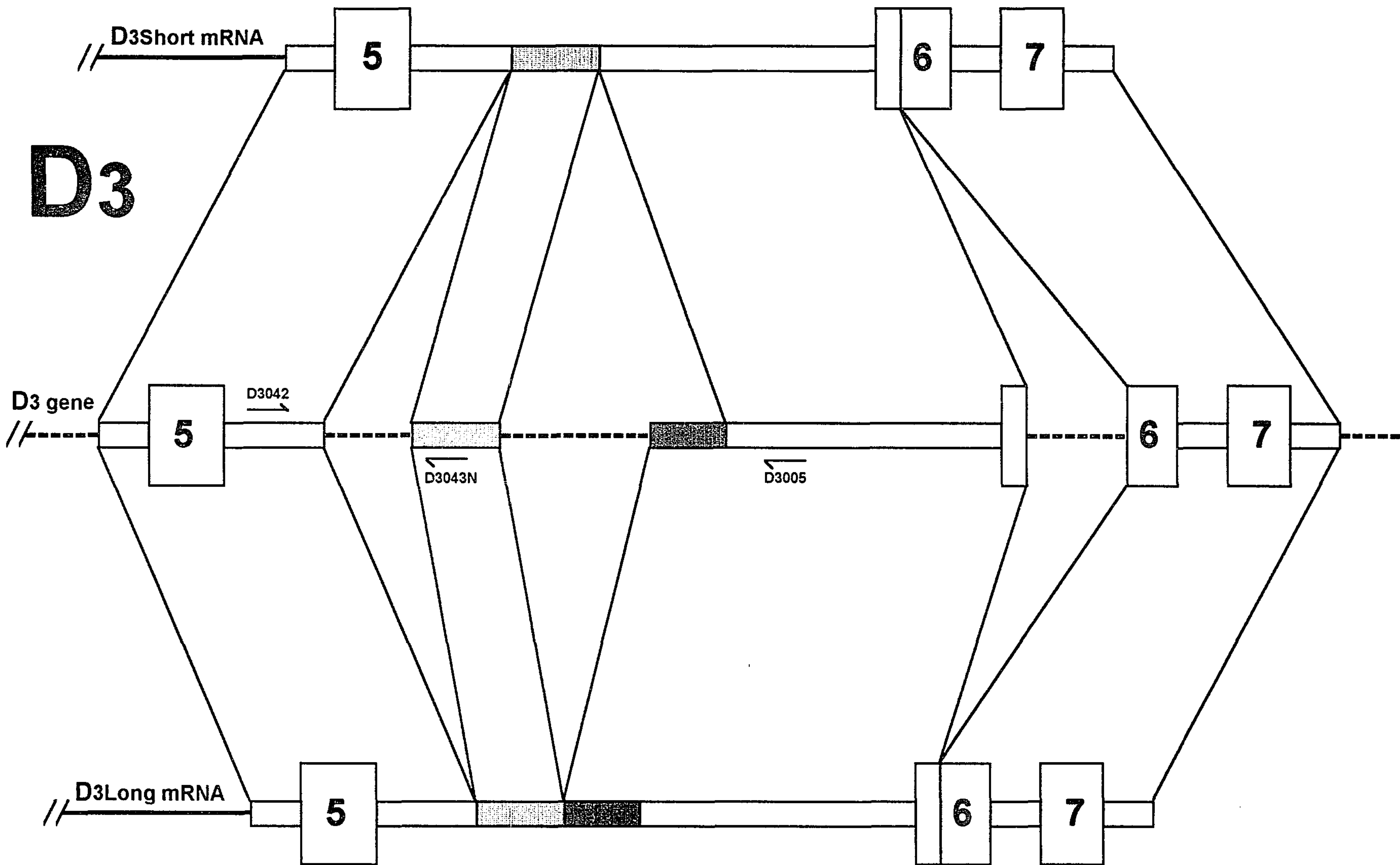

D_{3Short}
5'-3'(+)
G A T C

Figure 11-3. Schematic comparison of the post-transcriptional alternative splicing between the dopamine D_2 and D_3 receptor mRNAs. (pages 80 and 81)

Boxed areas are exons with the widened parts representing the *trans*-membrane domains (TM) 5, 6 and 7 as marked. Dotted lines are introns. Between TM 5 and TM 6 lies the pitative third intracellular loop encoding region, within which a small cassette exon (in red) is alternatively spliced only in the D_2 mRNA, while a 5' segment (in green) of the following exon is alternatively spliced only in the mouse D_3 mRNA.


D2Short mRNA
5
6
7
D2
D2 gene
5
6
7
D2Long mRNA
5
6
7


D3Short mRNA
5
6
7
D3
D3 gene
5
D3042
D3043N
D3005
6
7
D3Long mRNA
5
6
7

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