MOLECULAR CHARACTERIZATION OF G-PROTEIN COUPLED RECEPTORS

by

Qun-Yong Zhou

A Dissertation

Presented to the Department of Biochemistry and Molecular Biology
Oregon Health Sciences University
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

May 1992

APPROVED:

[redacted]

(Professor in charge of Thesis)

[redacted]

(Chairman, Graduate Council)

[redacted]

(Chairman, Department of Biochemistry and Molecular Biology)

TABLE OF CONTENTS

ACKOWLEGEMENTS

I am grateful for the support given by the Vollum Institute. I would like to thank my mentor and friend Olivier for his advise and eucouragement and allowing me the freedom to work independently and freely. I owe additional thanks to Bob, Claudia, Dave, Huub, Kari, Jim, John, Lisa, Paul and Yuan, with whom I have worked side-by-side for the past three and half years. My appreciation also goes to the members of my advisory committee: Drs. Clinton, Cone, Forte and Ullman. I would also like to say special thanks to my wife Chuanyu. Her love and support is indispensable to my career.

This thesis is dedicated to my parents, Aiqin and Fong Zhou.

Abstract

Dopamine plays an important role in motor, emotion control and cognition function of the central nervous system. Disturbances of dopaminergic neurotransmission are thought to contribute to etiology and/or pathology of neurological and psychiatric disorders. Molecular, biochemical, physiological and pharmacological studies have shown that dopamine exerts its diverse physiological functions through interaction with several dopamine receptors. The predominant ones are the D1 and D2 receptors. The D1 receptor is linked to the stimulation of adenylate cyclase and may also activate phospholipase C and Ca^{++} mobilization. By using two degenerate primers corresponding to putative receptor transmembrane segments III and VI, we have selectively amplified five rat striatal cDNA sequences that encode novel G protein-coupled receptors. We have characterized recently one of these clones (R226) as a new member of adenosine receptors: the A3 adenosine receptor. The A3 adenosine receptor may play a role in spermatogenesis. Another one, named R213, was identified as D1 dopamine receptor. We have cloned genes or cDNAs for the D1 dopamine receptor from species including man, rat and mouse. The cloned human D1 dopamine receptor gene has been characterized on the basis of four criteria: the deduced amino acid sequence, which reveals that it is a G protein-coupled receptor; the tissue distribution of its messenger RNA, which is compatible with that of the D1 dopamine receptor; its pharmacological profile when transfected into COS-7, Ltk^- and GH4 cells; and its ability to stimulate the accumulation of cyclic AMP in transfected cells. The human D1 receptor gene is 86% identical to the rat gene at the nucleotide level and 92% at amino acid sequence level. The partial mouse D1 receptor gene is almost identical to its rat homologue. The D1 receptor gene has been localized to long arm of human chromosome 5 at 5q35.1. We have delineated that the rat D1 receptor gene is organized into two exons

separated by a small intron in the 5' untranslated region of its mRNA. The transcription start site is located 864 base pairs upstream from the translational initiation site. The 5'-flanking region of rat D1 receptor does not contain TATA and CAAT canonical boxes, but has a high G+C content, potential CRE and GRE sequences, and binding sites for transcription factors such as Sp1, Ap1 and Ap2. Transfection studies with D1-CAT fusion genes have demonstrated that: the D1 promoter is active and information contained within 735 base pairs of 5'-flanking region appears to be sufficient to confer its cell-specific expression; and the D1 promoter responds to cAMP induction, suggesting the existence of an autoregulation mechanism by which the stimulation of D1 receptor exerts a positive feedback on its own gene expression.

INTRODUCTION

A. G PROTEIN-COUPLED RECEPTORS

Transmembrane signaling allows the cell to process and respond to a variety of extracellular information. Much of this information is provided in the form of concentration changes of regulatory molecules such as neurotransmitters, hormones, growth factors and local modulators. These extracellular ligands interact with membrane receptors, and the binding event is transduced into an intracellular signal. Several families of cell surface receptors including growth factor receptors, ligand-gated ion channels and G protein-coupled receptors have been identified that are coupled to different mechanisms of signal transduction.

The G protein-coupled receptor family is one of the major transmembrane signaling molecules. They mediate the actions of extracellular signals as diverse as light (2), odorants (3), peptides, and neurotransmitters (4). In the unicellular eucaryotic organism yeast, interaction of pheromones with their receptors (G protein-coupled receptors) triggers a program of developmental processes (5). In the higher vertebrates, G protein-coupled receptors have been identified for most of the neurotransmitters, hormones, and bioactive peptides (4).

Unlike growth factor receptors and ligand-gated ion channels, G protein-coupled receptors have no built-in effectors. Transmembrane signaling systems of G protein-coupled receptor consist of three membrane-bound protein components: (a) a cell surface receptor; (b) an effector, such as the adenylate cyclase or phospholipase C or an ion channel; (c) a G protein that is coupled to both the receptor and its effector. G proteins, ubiquitous for all eucaryotic cells, consist of three subunits (designated as α, β and γ). Multiple genes encoding the three subunits of G proteins have been isolated (1). The following model describes how signal transduction is thought to happen. Following the recognition of a receptor by its ligand, a conformational change of the receptor is transmitted to the G protein, which causes the α-subunit to exchange a bound GDP to GTP and to disassociate from the $\beta\gamma$-subunits. A single ligand-receptor complex can activate multiple G protein molecules, thus amplifying the ligand-receptor binding event. The α subunit bound with GTP and the free $\beta\gamma$ subunit may interact with effectors such as adenylate cyclase, phospholipase C, phospholipase A2, phosphodiestase, and ion channels, further amplifying the signal. Activity-modified ion channels and low molecular weight second messengers such as cAMP and inositol triphosphate then generate intracellular changes including membrane depolarization or hyperpolarization, protein phosphorylation, gene transcription and secretion. Termination of signaling occurs when the GTP of G protein a subunit is hydrolyzed to

GDP.

The bovine rhodopsin protein was purified and sequenced in 1983. Hydrophobicity analysis of its amino acid sequence using Kyte and Doolittle method (7) revealed that it had a structural profile remarkably similar to that of bacteriorhodopsin, a light-driven proton pump (8). Both bacteriorhodopsin and rhodopsin contain seven stretches of hydrophobic amino acids that represent possible membrane spanning domains. Image reconstruction from electron microscopy by Henderson and Uwin (10) provided a three-dimensional structure model for bacteriorhodopsin featuring seven α-helical transmembrane segments. The seven transmembrane structural motif and its α-helical nature of the individual transmembrane segment of bacteriorhodopsin and rhodopsin have been further supported by a variety of studies utilizing physical methods, chemical modification and proteolytic mapping (2).

Tremendous purification efforts on adrenergic receptors and muscarinic receptors finally led to the cloning of β2, α_2 and α1 adrenergic receptors and M1 and M2 muscarinic receptors (11-15). These findings were immediately followed by the cloning of substance K receptor and 5-HT1c receptor through Xenopus oocyte expression system (16-17). Gene or cDNA sequence comparison of these receptors and rhodopsin revealed that they are structurally related to one another in (a) a

strikingly similar overall structure motif: they all contain seven stretches of 20-28 hydrophobic amino acids, which likely represent membrane spanning regions; (b) their primary sequences: they bear significant similarity at both nucleotide and amino acid levels, especially in the putative membrane spanning regions and about a dozen amino acid residues are highly conserved in all of them. Thus, a concept arose which proposed that G protein-coupled receptors belong to a large gene family sharing the same seven transmembrane segment topography and sequence similarities with one another. Accordingly, bacteriorhodopsin or rhodopsin-like topography was proposed for the β2-adrenergic receptor and other G protein-coupled receptors (11,14). Limited proteolysis studies and immunolocalization (extracellular or cytoplasmic) using antipeptide antibody supported such a structural model of seven transmembrane segment topographic arrangement for β2 adrenergic receptor(18-19).

The existence of significant similarity at primary nucleotide sequence level among cloned G protein-coupled receptors immediately suggested a cloning strategy for new G protein-coupled receptors, referred to as low stringency screening. This approach is based on the observation that under reduced hybridization stringency conditions, sequences that are similar but not identical to the probe will be detected. For instance, we may cross-hybridize β1-adrenergic receptor cDNA or gene using β2 receptor clone as a probe to screen genomic

or cDNA libraries. By using the cloned receptor genes or cDNA as probes to screen libraries under low stringency hybridization conditions, several new members of G protein-coupled receptors have been successfully cloned including the 5HT1a receptor (20-22), dopamine D2 receptor (23), β1 and β3 adrenergic receptors(24-25), 5-HT2 receptor(26), M3-M5 muscarinic receptors(27-28) and substance P receptor (29). Indeed the successful cloning of these new members of G protein-coupled receptors using the low stringency screening method has not only revealed the existence of subfamilies (e.g. adrenergic receptor and muscarinic receptors) within the G protein-coupled receptor family but also reinforced the concept that G protein-coupled receptors share the same seven transmembrane topography and sequence similarity with one another. The concept of sequence similarity among G protein-coupled receptors is also the basis of our assumption for PCR-cloning of new members of G protein-coupled receptors including the dopamine D1 receptor (30).

B. Dopamine and dopamine D1 receptors

Dopamine plays an important role in motor control, affective and cognitive functions of the brain. The two major central dopaminergic pathways are: 1) the nigrostriatal system: which originates in pars compacta of substantia nigra (A9 cells) and projects through the medial forebrain bundle mainly to the neostriatum and nucleus accumbens, and to a lesser

extent, to the amygdaloid complex and some cortical areas. This dopaminergic pathway, which accounts for 70% of the total brain dopamine content, participates in the initiation and execution of movement. Loss of dopaminergic neurons of this dopaminergic pathway leads to Parkinson's symptoms such as involuntary tremor, akinesia, and rigidity (31). Depletion of dopamine in the striatum by either surgical or pharmacological means(such as the selective toxin for dopamine neurons, 1-methyl-4-phenyl-1,2,3,6-tetrohydropyridine, MPTP) will result in deficiency of movement including the symptoms of Parkinson's disease (32). L-dopa, a precursor of dopamine biosynthesis, provides a replacement therapy for Parkinson's disease. 2) the mesolimbic-mesocortical system: originates in the ventral tegmental area (A10 cells) and innervates mainly the prefrontal cortex, nucleus accumbens, amygdaloid complex and olfactory tubercle. This system is implicated in emotional control and cognitive function as well as locomotion control of human brain. The dopamine hypothesis of schizophrenia invokes enhanced activity of this mesolimbic-mesocortical dopaminergic pathway and dopamine receptor antagonists ameliorate the symptoms of schizophrenia (33). In rodents, behaviors such as self-stimulation, stereotypy, eating and drinking are attributed to this dopaminergic pathway.

Dopamine exerts its physiological effects through interacting with two classes of receptors, the D1 and D2 receptors (34).

These receptors can be differentiated physiologically and pharmacologically and by their anatomical distributions. Pharmacologically the hallmark of D1 receptors is to bind the benzazepine antagonist SCH23390, while that of D2 receptors is to recognize spiperone and haloperidol with high affinity (35). SKF38393 and quinpirole are selective agonists for dopamine D1 and D2 receptors respectively (35). D2 receptors are located either postsynapticly or presynapticly while D1 receptors are found exclusively postsynapticly. The distribution of dopamine D1 and D2 receptors in the brain has been extensively examined by binding assays and quantitative autoradiography (36). Using [^{3}H]SKF83566 or [^{125}I]SCH23390, high concentrations of D1 receptors can be demonstrated in the caudate putamen, nucleus accumbens, olfactory tubercle, substantia nigra pars reticulata, median amygdaloid nucleus, and cortex (36).

Ligand binding studies have shown that the affinity of agonists for both D1 and D2 receptors is altered by GTP, indicating that the G proteins probably mediate the transduction from agonist bound receptor to cellular response (37). The D1 receptors interact with Gs to activate adenylate cyclase, while the D2 receptors interact with Gi to inhibit adenylate cyclase (35). Preincubation of agonists results in a diminished effect in response to subsequent exposure to agonists, a phenomenon known as desensitization (38). For D1 receptors preincubation with dopamine results in a diminished

stimulation of adenylate cyclase in response to subsequent exposure to dopamine (38a, 38b). The desensitization of D1 receptors has been studied with brain slices and cloned NS20Y cells (38a,38b). The desensitization of D1 receptors may involve a functional uncoupling in addition to receptor down-regulation (38b).

For a long period of time, the behavioral effects of dopaminergic agents were attributed to D2 receptor while the role of D1 receptor was less clear. Recent studies have suggested that D1 receptor plays important roles in motor control (39), cognitive function (40), neuroendocrine effects (41-44), central nervous system development (45-47), and modulating the activity of D2 receptors (39, 48-50). In addition, D1 receptor mediates the activation of immediate-early genes by psychomotor stimulants and may be involved in nicotine addiction (50a-50c). Electrophysiological studies have demonstrated that D1 receptors indirectly decrease the firing rate of midbrain dopamine A9 and A10 neurons (48,50d-50e).

Receptor subtype heterogeneity of dopamine D1 receptor has been suggested by recent studies. Mailman and coworkers found that amygdaloid D1 receptor is not linked to adenylate cyclase and has lower affinity for SCH23390 compared to the prototypical D1 receptor (50f). Mahan et al reported that striatal D1 receptor can stimulate PI turnover and Ca^{++}

mobilization (50g). Currently, it is unknown whether a single receptor subtype can couple to adenylate cyclase or phospholipase C or if multiple receptor subtypes couple selectively to adenylate cyclase and phospholipase C. Cloning studies will be needed to elucidate the molecular heterogeneity of D1 receptors.

D1 receptors have been solubilized and partially purified (51). The binding subunit of D1 receptor has a molecular weight of approximately 72 KD, as determined by photoaffinity cross-linking (52). A similar molecular weight of about 79 KD was observed by radiation inactivation analysis. However, the primary structure of the D1 receptor at that time remained unknown. Our goal was to isolate the gene and cDNA for the dopamine D1 receptor and then to characterize the properties of the D1 receptor at molecular level using the cloned gene.

C. Adenosine and adenosine receptors

Adenosine modulates a variety of physiological functions through interacting with cell surface membrane proteins known as adenosine receptors (53). In the cardiovascular system, adenosine suppresses cardic rate and contractility. Adenosine is a vasodilator in almost all vascular beds. In the CNS, adenosine inhibits neurotransmitter release, produces sedation, and displays anticonvulsant activity. Adenosine

also participates in the regulation of metabolism by stimulating glyconeogenesis and inhibiting lipolysis.

Synaptic activation elicits the release of adenosine and ATP at numerous loci in the peripheral and central nervous system (53a). Once released, adenosine and ATP exert significant effects on the microphysiology of individual synapses and target organs. For example, adenosine and ATP inhibit the release of excitatory transmitters at most synapses and they may serve as negative feedback modulators of transmitter release (53a). Such synaptic actions are reflected at the gross behavioural level in the sedative and hypnotic effects of centrally-administrated adenosine (53a).

There are two pathways of biosynthesizing adenosine monophosphate (AMP): 1). De novo biosynthesis of AMP from amino acids (glycine, aspartate and glutamate), tetrahydrofolate derivatives, CO2 and PRPP (phosphoribosylpyrophosphate) through multiple enzymatic reactions (Fig.1). 2). Salvage synthesis of AMP from free adenine base, which is catalysed by the enzyme adenine phosphoribosyl transferase (Adenine + PRPP ----> AMP + PPi). AMP can be converted to ATP through the actions of adenylate kinase and nucleoside diphosphate kinase (Fig.2). Adenosine can be formed by the hydrolysis of AMP via 5'-nucleotidases (Fig.2).

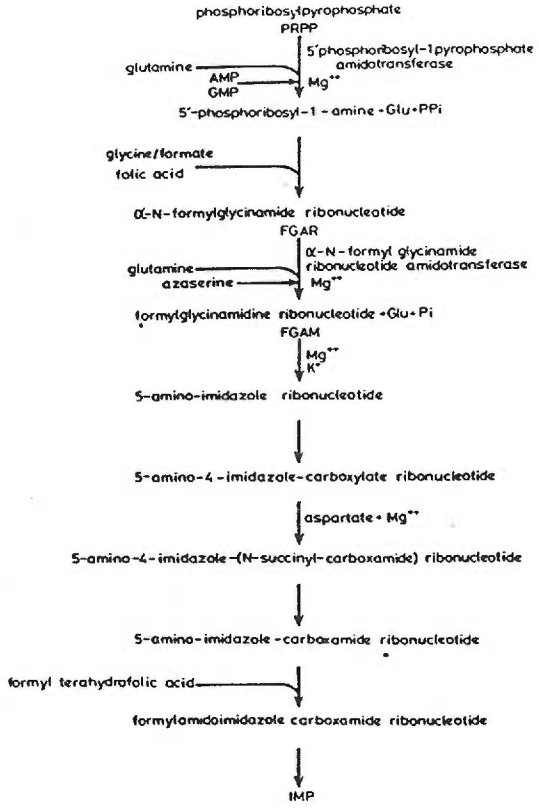


FIG. 1. Summary of the biosynthetic pathways involved in the *de novo* synthesis of purines. Starting materials include small molecular weight compounds such as glycine and glutamine, and the first purine product of the sequence is inosine 5'-monophosphate (IMP).

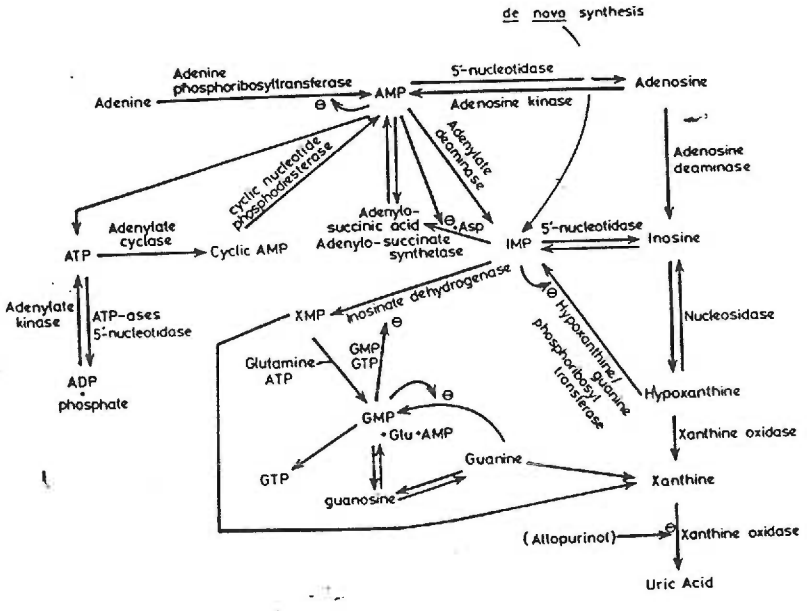


FIG. 2. A summary of some of the main metabolic interrelationships between purines.

Adenosine may be released by nerve stimulation from presynaptic or postsynpatic sources through a calcium-dependent process (53a). For example, adenosine has been suggested to be an inhibitory neurotransmitter in the guinea-pig myenteric plexus and cat vesical ganglion (53b, 53c). Adenosine in the synaptic milieu may also be derived from hydrolysis of ATP (released from synaptic vesicles) in the synaptic cleft via ecto-5'-ATPases and ecto-5'-nucleotidases (53a, 53d). The action of adenosine is terminated by uptake into cells via a dipyridamole-sensitive carrier (53a). Once inside the cells, adenosine is degraded to inactive nucleoside (inosine) by adenosine deaminase (Fig.2).

The classification of adenosine receptors into A1AR and A2AR has been widely accepted (54). At the A1AR, the potency order of synthetic adenosine analogues is R-PIA > NECA > S-PIA while at the A2AR, the order is NECA > R-PIA > S-PIA (55). A1AR and A2AR also differ in their sensitivity to the endogenous ligand adenosine. DPCPX is very selective antagonist ligand for A1AR while the only selective ligand available for A2AR is CGS21680, which is an agonist ligand (56-57).

Modulation of adenylate cyclase activity was the first biochemical effect of adenosine or its analogues to be described (58), and the abilities of A1AR and A2AR activation to respectively inhibit or stimulate adenylate cyclase

activity, provided an early ditinction between receptor subtypes (59-60). A1AR can also be coupled to the opening of K^+ channels and the blocking of Ca^{++} channels without the involvment of adenylate cyclase (61). The presynaptic inhibitory action of adenosine including inhibiting adenylate cyclase, opening K^+ channels and reducing flux through Ca^{++} channels is probably the mechanism by which adenosine inhibits neurotransmitter release (61). There are reports regarding the effects of adenosine on PI turnover, but these finding are considered controversial (62). All these activities of adenosine receptors appear to be mediated by G proteins based on its guanine nucleotide dependency and its sensitivity to pertussis toxin and cholera toxin. Incubation of A1AR and A2AR with their agonists resulted in their desensitization, i.e. a diminished inhibition or stimulation of adenylate cyclase in response to subsequent exposure to adenosine agonists (63-64). The mechanisms for the desensitization seem different for A1AR and A2AR with the nature being homologous for A2AR and heterologous for A1AR (63,65).

A1AR has been purified to homogeneity from the rat and bovine brains as well as from the testes (66-68). A1AR is a glycoprotein as determined by endo- and exo-glycosidase treatment of the purified receptor (67,69). In its deglycosylated state, A1AR is a 32 kD protein (66-67). The unavailability of selective A2AR antagonist ligands partially

results in the lag of A2AR research relative to A1AR with respect to biochemical mechanisms and receptor purification. The mass of the A2AR binding subunit (45 KD) has been determined recently by photoaffinity labeling with [^{125}]I-PAPA-APEC followed by SDS-PAGE (70). A2AR has yet to be purified to homogeneity.

Recently the cDNAs that encode A1AR and A2AR have been cloned by a PCR-based strategy (71-75). Molecular cloning of A1AR and A2AR revealed that they are proteins of 326 and 411 amino acids and they both belong to the G protein-coupled receptor superfamily. When transfected into COS-7 or CHO cells, their pharmacological characteristics were what expected for A1AR and A2AR.

However, recent pharmacological and physiological studies have suggested the existence of adenosine receptor heterogeneity beyond the A1AR and A2AR classification. Based on their affinity for [^{3}H]NECA, a nonselective adenosine receptor ligand, it has been proposed that A2AR may be divided into the A2a subtype (high affinity) or A2b subtype (low affinity) (76). Additional evidence for the existence of subtypes stems from the fact that A1 receptors are coupled to a large number of effectors in various tissues, including adenylate cyclase, atrial muscarinic-activated K^{+} channels, ATP-sensitive K^{+} channels, Ca^{++} channels and both stimulation and inhibition of phospholipase C (62). Moreover, A1

receptors appear to be coupled to multiple G proteins (62). Presently, it is unknown whether receptor subtypes couple selectively to various G proteins or if a single receptor can couple to multiple G proteins. Functional characterization of the cloned adenosine receptor cDNAs will help to solve this issue. Furthermore, Ribeiro and Sabastiao have suggested that there is a distinct adenosine receptor in the brain that is coupled to Ca^{++} metabolism (not related to adenylate cyclase) but no definitive characterization was provided (77). We present the cloning and functional characterization a novel adenosine receptor: the A3 adenosine receptor (manuscript #3 of this thesis).

References

1. Simon, S. I., Strathmann. M. P., & Gautam, N. Diversity of G proteins in signal transduction. *Science* **252**, 802-808, 1991.

2. Nathans, J. Molecular biology of visual pigments. *Annu. Rev. Neurosci.* **10**, 163-194, 1987.

3. Buck, L. & Axel, R. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* **65**, 175-187, 1991.

4. Dohlman, H. G., Thorner, J., Caron, M. G. & Lefkowitz, R. J. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**, 653-688, 1991.

5. Blumer, K. J. & Thorner, J. Receptor-G protein signaling in yeast. *Annu. Rev. Physiol.* **53**, 37-57, 1991.

7. Kyte, J. & Doolittle, R. F. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132, 1982.

8. Ovchinnikov, Yu. A., Abdulaev, N. G., Feigina, M. Yu., Artamonov, I. D., Bogachuk, A. S., Zolotarev, A. S., Eganyan, E. R., & Kostetskii, P. V. Visual rhodopsin III: complete amino acid sequence and topography in the membrane. *Bioorg. Khim.* **9**, 1331-1340, 1983.

9. Nathans, J. & Hogness, D. S. Isolation, sequence analysis and intron-exon arrangement of the gene encoding bovine rhodopsin. *Cell* **34**, 807-814, 1983.

10. Henderson, R. & Unwin, P. N. T. Three dimensional model of purple membrane obtained by electron microscopy. *Nature* **257**, 28-32, 1975.

11. Dixon, R.A.F., Koblika, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., Frielle, T., Bolanowski, M., Bennett, C., Rands, E., Diehl, R., Mumford, R., Slater, E., Sigal, I., Caron, M., Lefkowitz, R., Strader, C. Cloning of the gene and cDNA for mammalian β-adrenergic receptor receptor and homology with rhodopsin. *Nature* **321**, 75-79, 1986.

12. Kobilka, B. K., Matsui, H., Kobilka, T.S., Yang-Feng, T. L., Francke, U., et al. Cloning, sequencing and expression of the gene coding for human platelet α2-receptor. *Science* **238**, 650-656, 1987.

13. Cotecchia, S., Schwinn, D. A., Randall, R.R., Lefkowitz, R. J., Caron, M. G., & Kobilka, B. K. Molecular cloning and expression of the cDNA for hamster alpha1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **85**, 7159-7163, 1988.

14. Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi,

H., et al. Cloning, sequencing and expression of the complementary DNA encoding the muscarinic acetylcholine receptor. *Nature* **323**, 411-416, 1986.

15. Kubo, T., Maeda, A., Sugimoto, K., Akiba, I., Mikami, A., et al. Primary structure of porcine cardic muscarinic acetylcholine receptor deduced from the cDNA sequence. *FEBS lett.* **209**, 367-372, 1986.

16. Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M., & Nakanishi, S. cDNA cloning of bovine substance K-receptor through oocyte expression system. *Nature* **329**, 836-838, 1987.

17. Julius, D., MacDermott, A. B., Axel, R. & Jessell, T. M. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. *Science* **241**, 558-564, 1988.

18. Dohlman, H. G., Bouvier, M., Benovic, J. L., Caron. M. G. & Lefkowitz, R. J. The multiple membrane spanning topography of the β2-adrenergic receptor. *J. Biol. Chem.* **262**, 14282-14288, 1987.

19. Wang, H-Y., Lipfert, L., Malbon, C. C. & Bahouth, S. Site-directed antipeptide antibodies define the topography of the β-adrenergic receptor. *J. Biol. Chem.* **264**, 14424-14431, 1989.

20. Kobilka, B. K., Frielle, T., Collins, S.,Yang-Feng, T., Kobilka, T. S., et al. An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins. *Nature* **329**, 75-77, 1987.

21. Fargin, A., Raymond, J. R., Lohse, M. J., Kobilka, B. K., Caron, M. G., Lefkowitz, R. J. The genomic clone G21 which resembles a β-adrenergic receptor sequence encodes the 5HT1a

receptor. *Nature* **335**, 358-360, 1988.

22. Albert, P. R., Zhou, Q. Y., Van Tol, H. H. M., Bunzow, R., Civelli, O. Cloning, functional expression and mRNA tissue distribution of the rat 5-HT1a receptor. *J. Biol. Chem.* **265**, 5825-5832, 1990.

23. Bunzow, J.R., Van Tol, H.H.M., Grandy, D.K., Albert, P. Salon, J., Christie, M., Machida, C., Neve, K.A., Civelli, O. Cloning and expression of a rat D2 dopamine receptor cDNA. *Nature* **336**, 783-787 (1988).

24 . Frielle, T.,Collins., Daniel, K. W., Caron, M. G., Lefkowitz, R. J. & Kobilka, B. K. Cloning of the cDNA for the human β1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84**, 7920-7924, 1987.

25. Emorine, L. J., Marullo, S., Briend-Sutren, M. M., Patey, G., Tate, K., et al. Molecular characterization of the human β3-adrenergic receptor. *Science* **245**, 1118-1121, 1989.

26. Julius, D., Huang,K. N., Livelli, T. J., Axel, R,. Jessell, T. M. The 5HT-2 receptor defines a family of structurally distinct but functionally conserved serotonin receptor. *Proc. Natl. Acad. Sci. USA* **87**, 928-932, 1990.

27. Peralta, E. G., Ashkenazi, A., Winslow, J. W., Smith, D. H., Ramachandran, J., Capon, D. J. Distinct primary structures, ligand-binding properties and tissue-specific expression of four muscarinic acetylcholine receptors. *EMBO J.* **6**, 3923-3929, 1987

28. Bonner, T. I., Young, A. C., Brann, M. R., Buckley, N. J. Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor gene. *Neuron* **1**, 403-410, 1988.

29. Yokota, Y., Sasai, y., Tanaka, K., Fujiwara, T., Tsuchida, K., et al. Molecular characterization of a functional cDNA for rat substance P receptor. *J. Biol. Chem.* **264**, 17649-17652, 1989.

30. Bunzow, J. B. Zhou, Q.-Y. & Civelli, O. 1992. Cloning of dopamine receptors: homology approach. *Methods in Neurosciences* Academic Press. New York. in press.

31. Hornykiewicz, O. Dopamine and brain function. *Pharmacol. Rev.* **18**, 925-964, 1966.

32. Schultz, W. R. Depletion of dopamine in the striatum as experimental model of Parkinsonism: direct effects and adaptive mechanisms. *Prog. Neurobio.* **18**, 121-166, 1982.

33. Seeman P. Dopamine receptors and the dopamine hypothesis of schizophrenia. *Synapse* **1**, 133-152, 1987.

34. Kebabian, J. W. & Calne, D. B. Multiple receptors for dopamine *Nature* **227**, 93-96, 1979.

35. Creese, I. Dopamine receptors. In *The receptors,* ed. Conn, P. M. (Academic, New York), Vol.**4** pp.171-212, 1986.

36. Boyson, S.J., McGonigle, P., Molinoff, P.B. Quantitative autoradiographic localization of the D1 and D2 subtypes of dopamine receptors in rat brain. *J. Neurosci.* **6**, 3177-3188 (1986).

37. Hess, E.J., Creese, I., in *Dopamine receptors, receptor biochemistry and methodology* I. Creese, C. M. Fraser, Eds.(Alan R. Liss, New York, 1987, pp. 1-27..

38. Sibley, D. R. & Lefkowitz, R. J. β-adrenergic receptor-coupled adenylate cyclase: biochemical mechanisms of

regulation. *Molec. Neurobio.* **1**, 121-154, 1987.

38a. Memo, M., Lovenberg, W. & Habauer, I. (1982) Agonist-induced subsensitivity of adenylate cyclase coupled with a dopamine receptor in slices from rat corpus striatum. *Proc. Natl. Acad. Sci. USA* **79**, 4456-4460.

38b. Barton, A. C. & Sibley, D. R. (1990) Agonist-induced desensitization of D1 dopamine receptors linked to adenylyl cyclase activity in cultured NS20Y neuroblastoma cells. *Molec. Pharmacol.* **38**, 531-541.

39. Clark, D. & White, F. J. (1987) Review: D1 receptor- the search for a function: a critical evaluation of the D1/D2 dopamine receptor classification and its functional implications. *Synapse* **1**, 347-388.

40. Sawaguchi, T. & Goldman-Rakic, P. (1991) D1 dopamine receptors in frontal cortex: involvment in working memory. *Science* **251**, 947-950.

41. Reubi, J.-C., Iverson, L. L. & Jessell, T. M. (1977) Dopamine selectively increase [3H]GABA release from slices of rat substantia nigra *in vitro*. *Nature* **268**, 652-654.

42. Girault, J. A., Spampinato, U., Sauaki, H. E., Glowinski, J. & Besson, M. J. (1986) *In vivo* release of [^{3}H]γ-aminobutyric acid in the neostriatum. *Neuroscience* **19**, 1101-1108.

43. Benkirane, S., Arbilla, S. & Langer, S. (1987) A functional response to D1 dopamine receptor stimulation in the central nervous system: inhibition of the release of [^{3}H]serotonin from the rat substantia nigra. *Naunyn-Schmiedebergs Arcg. Pharmacol.* **335**, 502-507.

44. Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z., Chase, T. N., Monsma, F. J., Jr.& Sibley, D. R. (1990) D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* **250**, 1429-1432.

45. Lankford, K. L., DeMello, F. G. & Klein, W. L. (1988) D1-type dopamine receptors inhibit growth cone mobility in cultured retina neurons: evidence that neurotransmitters act as morphogenic growth regulators in the developing central nervous system. *Proc. Natl. Acad. Sci. USA* **85**, 4567-4571.

46. Rodrigues, P. D. S. & Dowling, J. E. (1990) Dopamine induces neurite retraction in retinal horizontal cells via diacylglycerol and protein kinase C. *Proc. Natl. Acad. Sci. USA* **87**, 9693-9697.

47. Mattingly, B. A., Rowleff, J. K., Graff, J. T. & Hatton, B. J. (1991) Effects of selective D1 and D2 dopamine antagonists on the development of behavioral sensitization to apomorphine. *Psychopharmacology* **105**, 501-507.

48. Waters, J. R., Bergstrom, D. A., Carlson, J. H., Chase, T. & Braun, A. (1987) D1 dopamine receptor activation required for postsynaptic expression of D2 agonist effects. *Science* **236**, 719-722.

49. Waddington, J. L. (1989) Functional interactions between D1 and D2 dopamine receptor system: their role in the regulation of psychomotor behaviour, putative mechanisms and clinical relevance. *J. Psychopharmacol.* **3**, 54-63.

50. Bertorello, A. M., Hopfield, J. F., Aperia, A. & Greengard, P. (1990) Inhibition by dopamine of (Na^+-K^+)ATPase activity in neostriatal neurons through D1 and D2 dopamine receptor synergism. *Nature* **347**, 386-388.

50a. Acquas, E., Carboni, E., Leone, P. & DiChiara, G. (1989) SCH23390 blocks drug-conditioned place-preference and place-aversion: anhedonia (lack of reward) or apathy (lack of motivation) after dopaminergic blockage? *Psychopharmacology* **99**, 151-155.

50b. Graybiel, A. M., Moratalla, R. & Robertson, H. A. (1990) Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striasome-matrix compartments and limbic subdivisions of the striatum. *Proc. Natl. Acad. Sci. USA* **87**, 6912-6916.

50c. Young, S. T., Porrino, L. J. & Zadarola, M. J. (1991) Cocaine induces striatal c-Fos-immunoreactive proteins via dopaminergic D1 receptors. *Proc. Natl. Acad. Sci. USA* **88**, 1291-1295.

50d. Carson, J. H., Bergstrom, D. A., Weick, B. G. & Waters, J. R. Neurophysiological investigation of the effects of D1 agonist SKF38393 on tonic activity of substania nigra dopamine neurons. *Synapse* **1**, 411-416, 1987.

50e. Wachet, S. W. & White, F. J. Interaction of D1 and D2 receptors within the rat mesoaccumbens dopamine system: electrophysiological studies. *Soc. Neurosci. Abstr.* **12**, 1516, 1986.

50f. Mailman, R. B., Schulz, D. W., Kilts, C. D., Lewis, M. H., Rollema, H. & Wyrick, S. *Adv. Exp. Med. Bio.* **204**, 53-72, 1986.

50g. Mahan, L. C.,Burch, R. M., Monsma, F. J. & Sibley, D. R. Expression of striatal D1 dopamine receptors coupled to inositol phosphate production and Ca^{++} mobilization in Xenopus oocytes. *Proc. Natl. Acad. Sci. USA* **87**, 2196-2200, 1990.

51. Niznik, H. B., Grigoriadis, D. E., Otsuka, N. Y., Dumbrille-Ross, A. & Seeman, P. The dopamine D1 receptor: partial purification of a digitonin-solubilized receptor-guanine nucleotide binding complex. *Biochem. Pharmacol.* **35**, 2974-2977, 1986.

52. Amlaiky, N., Berger, J. G., Chang, W., McQuade, R. J. & Caron, M. G. Identification of the binding subunit of the D1 dopamine receptor by photoaffinity crosslinking. *Molec. Pharmacol.* **31**, 129-134, 1986.

53. Williams, M. Purine receptors in mammalian tissues: pharmacology and functional significance. *Annu. Rev. Pharmacol. Toxicol.* **27**, 315-345, 1987.

53a. Stone T. W. Physiological roles for adenosine and adenosine-5'-triphosphate in the nervous system. *Neurosci.* **6**, 523-555, 1981.

53b. Palmer, J. M., Wood, J. D. & Zafirov, D. H. Purinergic inhibition in the small intestinal myenteric plexus of the guinea-pig. *J. Physiol.* **387**, 357-370, 1987.

53c. Akasu, T., Shinnick, P. & Gallagher, J. P. Actions of purines in autonomic ganglia, in Purines: Pharmacological and physiological Roles. (Stone, T. W. ed), pp 57-66, MacMillan, London, 1985.

53d. Richardson, P. J., Brown, S. J., Bailyes, E. M., & Luzio, J. P. Ectoenzymes control adenosine modulation of immunoisolated cholinergic synapses. *Nature* **327**, 232-234, 1987.

54. Wolff, J., Londos, C.& Cooper, D. M. F. Adenosine receptors and the regulation of adenylate cyclase. In

advances in cyclic nucleotide research, ed. J. E. Dumont, P. Greengard, G. A. Robinson, **14**, 188-214. New York, Raven. 1981.

55. Stiles, G. L. (1986) Adenosine receptors: structure, function and regulation. *Trends Pharmacol. Sci.* **7**, 486-490.

56. Lohse, M. J., Klotz, K. N., Lindenborn-Fotinos, J., Reddington, M, Schwabe, U. & Alsson, R. DPCPX-a selective high affinity antagonist radioligand for A1 adenosine receptors. *Naunyn-Schmiedeberg's Arch Pharmacol.* **336**: 204-210, 1987;

57. Jarvis, M. F., Schulz, R., Hutchison, A. J., Do, U. H., Sills, M. A. & Williams, M. [^{3}H] CGS21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brains. *J. Pharmacol. Exp. Ther.* **251**, 888-893, 1989.

58. Sattin, A. & Rall, T. W. The effect of adenosine and adenine nucleotides on the cyclic adenosine 3',5'-phosphate content of guinea pig cerebral cortex slices. *Mol. Pharmacol.* **6**, 13-23, 1970.

59. Londos, C., Cooper, D. M. F. & Wolff, J. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA.* **77**, 2551-2554, 1980.

60. Van Calker, D., Muller, M. & Hamprecht, B. Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.* **33**, 999-1005, 1979.

61. Fredholm, B. & Dunwiddie, T. V. (1988) How does adenosine inhibit transmitter release? *Trends Pharmacol. Sci.* **9**, 130-134.

62. Linden J. Structure and function of A1 adenosine receptors. *FASEB J.* **5**, 2668-2676, 1991.

63. Parsons, W. J. & Stiles, G. L. Heterologous desensitization of the inhibitory A1 adenosine receptor-adenylate cyclase system in rat adipocytes: regulation of both Ni and Ns. *J. Biol. Chem.* **262**, 841-847, 1987.

64. Kenimer, J. G. & Nirenberg, M. Desensitization of adenylate cyclase to prostaglandin E1 or 2-choloradenosine. *Mol. Pharmacol.* **20**, 585-591, 1981.

65. Anand-Srivastava, M. B., Cantin, M., Ballak, M. & Picard, S. Desensitization of the stimulatory A2 adenosine receptor-adenylate cyclase system in vascular smooth muscle cells from rat arota. *Molec. Cell Endocrino.* **62**, 273-279, 1989.

66. Nakata, H. Purification of A1 adenosine receptor from rat brain membranes. *J. Biol. Chem.* **264**, 16545-16551, 1989.

67. Olah, M. E., Jacobson, K. A. & Stiles, G. L. (1990) Purification and characterization of bovine cerebral cortex A1 adenosine receptor. *Arch. Biochem. Biophys.* **283**, 440-446.

68. Nakata H. A1 adenosine receptor of rat testis membranes: purification and partial characterization. *J. Biol. Chem.* **265**, 671-677, 1990.

70. Barrington, W. W., Jacobson, K. A. & Stiles, G. L. Glycoprotein nature of the A2 adenosine receptor binding subunit. *Mol. Pharmacol.* **38**, 177-183, 1990.

71. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M., Dumont, J. E. & Vassart, G. (1989) Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science*

244, 569-572.

72. Maenhaut, C., Van Sande, J., Libert, F., Abramowicz, M., Parmentier, M., Vanderhaegen, J. J., Dumont, J. E., Vassart, G. & Schiffmann, S. (1990) RDC8 codes for an adenosine receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* **173**, 1169-1178.

73. Reppert. S. M., Weaver, D. R., Stehle, J. H. & Rivkees, S. A. (1991) Molecular cloning and characterization of a rat A1 adenosine recptor that is widely expressed in brain and spinal cord. *Mol. Endocrin.* **5**, 1037-1048.

74. Libert, F., Schiffmann, S. N., Lefort, A., Parmentier, M., Gerard, C., Dumont, J. E., Vanderhaeghen, J. J. & Vassart, G. (1991) The orphan receptor cDNA RDC7 encodes an A1 adenosine receptor. *EMBO J.* **10**, 1677-1682.

75. Mahan, J. C., McVittie, L. D., Smyk-Randall, E. M., Nakata, H., Monsma, F. J., Jr., Gerfen, C. R. & Sibley, D. R. (1991) Cloning and expression of an A1 adenosine receptor from rat brain. *Molec. Pharmacol.* **40**, 1-7.

76. Brauns, R. F., Lu. G. H. & Pugsley, T. A. Characterization of the A2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membrane. *Mol. Pharmacol.* **29**, 331-346, 1986.

77. Ribeiro, J. A. & Sebastiao, A. M. (1986) Adenosine receptors and calcium: basis for proposing a third adenosine receptor. *Prog. Neurobiol.* **26**, 79-209.

Introduction

Design of oligonucleotides

Amino acid sequence alignment of cloned G protein-coupled receptors revealed high conservation of primary sequence in putative transmembrane domains III and VI, especially among catecholamine receptors (1). To clone the D_1 receptor, we designed the following pair of degenerate oligonucleotides taking the advantage of nucleotide sequence similarity among cloned catecholamine receptors and other known amine receptors. Sometimes, inosine was used to reduce degeneracy.

TM

III GAGTCGACCTGTGCGCCATCACIITGGACCGCTAC 5' oligonucleotide

(Sal I) T TG TG TA G

β_1	CTGTGTGTCATTGCCCTGGACCGCTAC
β_2	CTGTGCCTCATCGCAGTGGATCGCTAC
α_2	CTGTGCGCCATCAGCCTGGACCGCTAC
D_2	CTGTGTGCCATCAGCATTGACAGGTAC
$5HT_{1a}$	CTGTGCGCCATCGCGCTGGACAGGTAC
m1	CTGCTGCTCATCAGCTTTGACCGCTAC

TM

VI CAGAATTCAGAAGGGCAICCAGCAGAIGGTGAA 3' oligonucleotide

(EcoRI) T CAC

β_1	AGAAGGGCAGCCAGCAGAGCGTGAA
β_2	AGAAGGGCAGCCAGCAGAGGGTGAA
α_2	AGAAGGGGAACCAGCACACCACGAA
D_2	AGAAGGGCAGCCAGCAGATGATGAA
$5HT_{1a}$	AGAAGGGCAGCCAGCAGAGGATGAA
m1	TGTACGGCGGCCAGGAGACGATGAA

(I: inosine; TM: transmembrane domain)

To facilitate the subsequent subcloning experiments, restriction enzyme cleavage sites have been added at the end of each oligonucleotide.

Polymerase chain reaction

Striatum was chosen as the source of template mRNA because radioligand binding studies and receptor autoradiography have shown the highest expression level of dopamine D_1 receptor in this tissue (2). Striata were dissected from male Sprague-Dawley rats. Total RNA was isolated using the guanidinium thiocyanate method (3). Poly A^+ mRNA was purified from total RNA by oligo dT affinity chromatography. Double-stranded cDNA was synthesized from poly A^+ mRNA using AMV reverse transcriptase (3).

The PCR conditions were chosen to allow for hybridization of cDNA sequences with a low level of identity to the oligonucleotide primers (4). Therefore the annealing temperature was low. These conditions can be applied for the cloning of any receptor. About 10 ng striatum cDNA was used as template in 30 cycles of PCR, consisting of one minute denaturation at 95°C, two minutes annealing at 45°C, and three minutes extension at 72°C in a solution of 100 µl containing 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris pH 8.4, 200 µM each dNTP, 1 µM of each degenerate oligonucleotide, and 2.5 units of Taq polymerase (Cetus).

Product purification and sequencing

Deletion studies have shown that the third cytoplasmic loop is crucial for G protein-coupling (5). The three cloned b-adrenergic receptors that coupled to G_s proteins have a third cytoplasmic loop of 52-78 amino acids (β_1, β_2, β_3), while the α_2 adrenergic and D_2 dopamine receptors that couple to G_i have loops of over 130 amino acids (α_2, D_2). Since the D_1 receptor couples to G_s protein, its third cytoplasmic loop was predicted to be of a size similar to that of β-adrenergic receptors. By using the primers described above, we anticipated that the PCR products encoding the dopamine D_1 receptor would be of about 550 bp.

The PCR products were digested with both EcoRI and SalI and

separated on a 1.2% agarose gel (Figure in appendices). Three contiguous gel slices corresponding to fragments ranging from 300-450, 450-700, and 700-1000 base pairs (bp) were excised from the gel. DNAs were purified from the gel slices with the Geneclean kit (Bio 101) and subcloned into EcoRI and SalI-digested M13mp18 and M13mp19. A total of 36 M13 recombinant clones were sequenced. Of them, 24 clones were from the 450-700 bp PCR products. As controls, we also sequenced 6 clones from each of the other gel slices. Preliminary sequence analysis showed that 16 out of these 36 templates encoded putative G protein-coupled receptor fragments. As discussed above we expected that the 700-1000 bp products would encode G_i-coupled receptor, and we indeed detected the dopamine D_2 as major part of these products. The small sized products (300-450 bp) should encode receptor with short third cytoplasmic loops, possibly receptor linked to inositol triphosphate turnover and we detected the substance P receptor and RDC4, which was recently identified as the 5-HT1d receptor (6). Of greater interest, the 450-700 bp products contained: 1) four new clones belonging to the G protein-coupled receptor superfamily named R21, R23, R222 and R226. R222 was the same as the recently cloned marijuana or cannabinoid receptor (7), and very recently we have identified R226 as the A3 adenosine receptor (manuscript #3 of this thesis) while the endogenous ligands for R21 and R23 are presently unknown. The partial sequence of R21, R23 and R222 can be found in the appendices of this thesis. 2) a 529

bp clone, R213. The R213 protein had a primary sequence which exhibited all the structure features we expected for dopamine D1 receptors: i). It had a higher degree of amino acid similarity with known catecholamine receptors than with other G protein-coupled receptors; ii). It contained an aspartic acid in the putative third transmembrane domain, which possibly act as counterion for the positive charge of catecholamine; iii). R213 contained two serine residues in the putative fifth transmembrane domain which are thought to be specific to receptors binding catecholamines; and iv). R213 had a putative third cytoplasmic loop similar in size and sequence to that of the β-adrenergic receptors. Our results of manuscript #1 have demonstrated that R213 encodes the dopamine D1 receptor.

Reference

1. Dohlman, H. G., Thorner, J., Caron, M. G. & Lefkowitz, R. J. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**, 653-688, 1991.

2. Boyson, S. J., McGonigle, P., Molinoff, P. B. Quantitative autoradiographic localization of the D1 and D2 subtypes of dopamine receptors in rat brain. *J. Neurosci.* **6**, 3177-3188, 1986.

3. Maniatis, T., *Molecular Cloning* (Cold Spring Harbor Press, Cold Spring Harbor, NY, 1990.

4. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491, 1988.

5. O'Dowd, B. F., Lefkowitz, R. J. & Caron, M. G. Structure of the adrenergic and related receptors. *Annu. Rev. Neurosci.* **12**, 67-83, 1989.

6. Hamblin, M. W. & Metcalf, M. A. Primary structure and functional characterization of a human 5-HT1d-type serotonin receptor. *Molec. Pharmacol.* **40**, 143-148, 1991.

7. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C. & Bonner, T. I. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346**, 561-564, 1990.

Cloning and Expression of a Human and a Rat D_1 Dopamine Receptors

Qun-Yong Zhou, David K.Grandy, Lisa Thambi, Jake Kushner, Hubert H. M. Van Tol*, Roger Cone, David Pribnow #, John Salon, James R. Bunzow and Olivier Civelli#+

Vollum Institute for Advanced Biomedical Research
and #Department of Cell Biology and Anatomy
Oregon Health Sciences University
3181 SW Sam Jackson Park Road
Portland, Oregon 97201

* Present address: Department of Pharmacology,
Medical Sciences Building,
University of Toronto,
Toronto, Ontario M5S 1A8,
Canada

+ To whom correspondence should be sent

Key Words: D1 dopamine receptor / cloning / expression

ABSTRACT

The importance of the dopaminergic system in brain function has been emphasized by its association with neurological and psychiatric disorders such as Parkinson's disease and schizophrenia. On the basis of their pharmacological and biochemical characteristics, dopamine receptors are classified into D1 and D2 subtypes[1,2]. As the most abundant dopamine receptor in the central nervous system, D1 receptors seem to mediate some behavioral responses[3], modulate activity of D2 dopamine receptors[4,5], and regulate neuron growth and differentiation[6]. The D2 dopamine receptor has been cloned by low-stringency screening[7]. We report here the cloning of human and rat D1 dopamine receptors by applying an approach based on the polymerase chain reaction[8]. The cloned human D1 receptor has been characterized on the basis of four criteria: the deduced amino-acid sequence, which reveals that it is a G protein-coupled receptor; the tissue distribution of its messenger RNA, which is compatible with that of the D1 dopamine receptor; its pharmacological profile when transfected COS-7 cells; and its ability to stimulate the accumulation of cyslic AMP in human 293 cells.

The D_1 and D_2 dopamine receptors are G-protein-coupled receptors that stimulate and inhibit adenylate cyclase, respectively[1,2]. The rat D_2 receptor was cloned by low stringency screening using the hamster β_2-adrenergic receptor as a probe[7]. To clone the D_1 receptor we applied a different approach. A set of degenerate oligonucleotide primers were designed based on the nucleotide sequences of known catecholamine receptors and some other G-protein-coupled receptors[9,10]. Two primers, corresponding to putative receptor transmembrane domains III and VI, were used in a polymerase chain reaction(PCR)[8]. Rat striatum cDNA was chosen as the template because high levels of D_1 dopamine receptor have been found in this tissue[11]. Deletion studies have shown that the third cytoplasmic loop is crucial for G-protein coupling[12]. Since the three cloned β-adrenergic receptors that couple to Gs have putative third cytoplasmic loops of 52-78 amino acids[13], we hypothesized that the third cytoplasmic loop of the dopamine D_1 receptor might be in a similar size range. Therefore, the PCR products were size-fractionated and products ranging from 450 to 700 bp were subcloned into M13 and subjected to direct sequencing. Of 24 PCR products analyzed, D_2 dopamine[7], α_{2B} adrenergic[14] and five sequences representing potentially new G-protein-coupled receptors were obtained. One of these clones, R213, had several interesting structural features. It had a higher degree of amino acid similarity with known catecholamine receptors as compared to other G-protein-coupled receptors; in the putative fifth

transmembrane domain it contained two serine residues which were thought to be specific to receptors binding catecholamines[15]; and it had a putative third cytoplasmic loop similar in size and sequence to that of the β-adrenergic receptors[13].

The PCR generated clone R213 was used as a probe to screen a rat striatum cDNA library. One positive clone was identified and sequenced. Although not full-length, it allowed us to describe most of the rat coding sequence (Fig.1). Since most catecholamine receptors lack introns in their coding regions[16] and since our preliminary human genomic analysis indicated the absence of introns in this gene (unpublished observations), we screened a human genomic library. Eight positive signals were obtained. One clone, HGR213-1, was further characterized and a 3.0 kb EcoRI/SacI fragment spanning the whole coding region was subcloned and sequenced.

Figure 1 shows the nucleotide sequence of clone HGR213-1. The longest open reading frame codes for a 446 amino-acid protein(relative molecular mass Mr= 49,296). This relative molecular mass is similar to the reported value of the deglycosylated form of dopamine D_1 receptor as determined by SDS-PAGE[17]. Like most adrenergic receptors, but unlike the dopamine D2 receptor[18], HGR213-1 has no intron in its coding sequence. There are two potential in-frame initiation sites. Considering the unique potential N-linked glycosylation site in the N-terminus, the initiation site shown in Fig.1 is most

likely the one which is used. Interestingly, there exists another potential N-linked glycosylation site in the second extracellular loop.

Hydrophobicity analysis of HGR213-1 revealed seven stretches of hydrophobic amino acids that could represent transmembrane domains(data not shown). Comparison of the deduced amino acid sequence of HGR213-1 with that of other catecholamine receptors shows that the greatest similarity exists in the putative transmembrane domains where the amino acid identities are as follows: 44% with human D_2[18], 42% with human β_2[19], 43% with human β_1[20], 41% with hamster α_1[21], 42% with human α_{2A}[22] and 40% with human α_{2B}[14]. The overall degree of identity between D_1 and D_2 receptors is about the same as between the D_1 and other catecholamine receptors. Asp79 and Asp113 in the β_2-adrenergic receptor, which possibly act as counterions for the positively charged catecholamine[23], are present at corresponding positions in HGR213-1. Furthermore, the size and sequence of its third cytoplasmic loop and C-terminus of HGR213-1 are similar to that of β-adrenergic receptors. This suggested to us that this new receptor might be coupled to Gs[12]. However, the absence of a potentially important glutamic acid residue[24], which is conserved in the third transmembrane domains of all three cloned β-adrenergic receptors[13] indicated that HGR213-1 probably was not a β-adrenergic-like receptor. Based on these structural features, we hypothesized that HGR213-1 could encode a dopamine D_1 receptor. In addition, there exist two consensus sequences

(residues 133-136, 265-268) for cAMP-dependent protein kinase phosphorylation[9] and many serines and threonines residing in the cytoplasmic loops and the relatively long C-terminus could be potential protein kinase C[25] or receptor kinase[26] phosphorylation sites.

As a step towards identifying HGR213-1, the tissue distribution of its transcript was examined by Northern blot analysis. As shown in Fig.2, a messenger RNA of approximately 4 kb was found in many rat brain regions with the highest level of expression in the basal ganglia. HGR213-1 mRNA was undetectable in the pituitary and in the peripheral tissues we tested. This pattern of HGR213-1 messenger distribution in the central nervous system and pituitary is consistent with that of the dopamine D1 receptor as determined by autoradiography and binding studies[11].

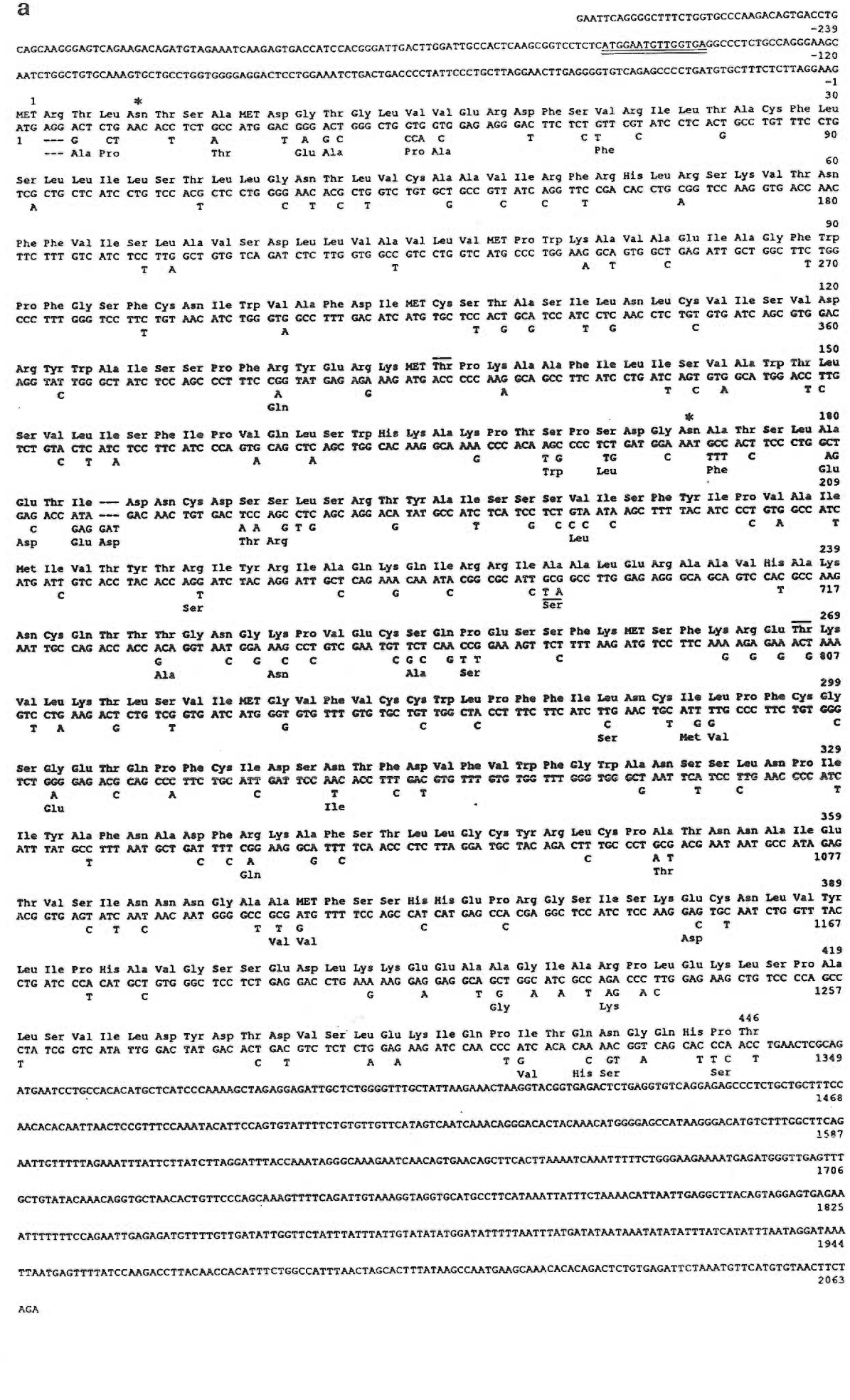
To further address its identity, HGR213-1 was transiently expressed in eukaryotic cells. The 3.0 kb EcoRI/SacI fragment of HGR213-1 was inserted into eukaryotic expression vector PBC12BI[27] and transfected into monkey kidney COS-7 cells. Since its structural features and mRNA tissue distribution suggested that HGR213-1 might encode a dopamine D_1 receptor, membranes from transfected COS-7 cells were tested for their ability to bind to the D1 selective antagonist [^{3}H]SCH23390. Untransfected COS-7 cells showed no specific binding of [^{3}H]SCH23390 (data not shown). Binding of [^{3}H]SCH23390 to membranes prepared from transfected COS-7 cells was saturable with a dissociation constant (Kd) of 0.3 nM (Fig.3 inset).

This value agrees well with both the reported value[29] and the value observed in parallel experiments with rat striatal membranes(data not shown). Fig.3 shows competition curves of various ligands with [^{3}H]SCH23390. The D_1 selective antagonist SCH23390 and agonist SKF82526 were most potent while the D_2 selective antagonist haloperidol was virtually inactive. The rank order of ligand potency was: SCH23390 > (+)Butaclamol > Flupenthixol >> Haloperidol. This pharmacological profile explicitly identifies the binding site as that of a dopamine D_1 receptor.

To demonstrate that HGR213-1 encodes a functional dopamine D_1 receptor we examined its ability to couple dopamine binding to activation of adenylate cyclase. Human embryonic kidney 293 cells transiently expressing HGR213-1 were tested for their ability to respond to dopamine. When exposed to dopamine, untransfected cells showed no elevation of cAMP (Fig.4a). In contrast, transfected cells displayed a concentration-dependent and saturable increase of intracellular cAMP levels with a half-maximal stimulation concentration (EC50) of about 125 nM (Fig.4a). This value is comparable to the reported value[29]. SKF38393, a selective D_1 agonist, had a similar effect on the intracellular cAMP production and the stimulatory effects of both dopamine and SKF38393 were blocked by SCH23390 (Fig.4b). These results indicated that the cloned D_1 receptor could couple positively to adenylate cyclase.

Based on the above results we conclude that HGR213-1 encodes a human D_1 dopamine receptor. The successful cloning of the human D_1 dopamine receptor provides a new tool to study the regulation and function of this receptor. Moreover, the availability of both D_1 and D_2 dopamine receptor clones, which both bind to dopamine but couple to distinct effectors, should provide us with a new approach to address the complex interactions between these receptors.

a


GAATTCAGGGGCTTTCTGGTGCCCAAGACAGTGACCTG
-239
CAGCAAGGGAGTCAGAAGACAGATGTAGAAATCAAGAGTGACCATCCACGGGATTGACTTGGATTGCCACTCAAGCGGTCCTCTCATGGAATGTTGGTGAGGCCCTCTGCCAGGGAAGC
-120
AATCTGGCTGTGCAAAGTGCTGCCTGGTGGGGAGGACTCCTGGAAATCTGACTGACCCCTATTCCCTGCTTAGGAACTTGAGGGGTGTCAGAGCCCCTGATGTGCTTTCTCTTAGGAAG
-1
1
30
MET Arg Thr Leu Asn Thr Ser Ala MET Asp Gly Thr Gly Leu Val Val Glu Arg Asp Phe Ser Val Arg Ile Leu Thr Ala Cys Phe Leu
ATG AGG ACT CTG AAC ACC TCT GCC ATG GAC GGG ACT GGG CTG GTG GTG GAG AGG GAC TTC TCT GTT CGT ATC CTC ACT GCC TGT TTC CTG
90
--- Ala Pro Thr Glu Ala Pro Ala Phe
60
Ser Leu Leu Ile Leu Ser Thr Leu Leu Gly Asn Thr Leu Val Cys Ala Ala Val Ile Arg Phe Arg His Leu Arg Ser Lys Val Thr Asn
TCG CTG CTC ATC CTG TCC ACG CTC CTG GGG AAC ACG CTG GTC TGT GCT GCC GTT ATC AGG TTC CGA CAC CTG CGG TCC AAG GTG ACC AAC
180
90
Phe Phe Val Ile Ser Leu Ala Val Ser Asp Leu Leu Val Ala Val Leu Val MET Pro Trp Lys Ala Val Ala Glu Ile Ala Gly Phe Trp
TTC TTT GTC ATC TCC TTG GCT GTG TCA GAT CTC TTG GTG GCC GTC CTG GTC ATG CCC TGG AAG GCA GTG GCT GAG ATT GCT GGC TTC TGG
270
120
Pro Phe Gly Ser Phe Cys Asn Ile Trp Val Ala Phe Asp Ile MET Cys Ser Thr Ala Ser Ile Leu Asn Leu Cys Val Ile Ser Val Asp
CCC TTT GGG TCC TTC TGT AAC ATC TGG GTG GCC TTT GAC ATC ATG TGC TCC ACT GCA TCC ATC CTC AAC CTC TGT GTG ATC AGC GTG GAC
360
150
Arg Tyr Trp Ala Ile Ser Ser Pro Phe Arg Tyr Glu Arg Lys MET Thr Pro Lys Ala Ala Phe Ile Leu Ile Ser Val Ala Trp Thr Leu
AGG TAT TGG GCT ATC TCC AGC CCT TTC CGG TAT GAG AGA AAG ATG ACC CCC AAG GCA GCC TTC ATC CTG ATC AGT GTG GCA TGG ACC TTG
Gln
180
Ser Val Leu Ile Ser Phe Ile Pro Val Gln Leu Ser Trp His Lys Ala Lys Pro Thr Ser Pro Ser Asp Gly Asn Ala Thr Ser Leu Ala
TCT GTA CTC ATC TCC TTC ATC CCA GTG CAG CTC AGC TGG CAC AAG GCA AAA CCC ACA AGC CCC TCT GAT GGA AAT GCC ACT TCC CTG GCT
Trp Leu Phe Glu
209
Glu Thr Ile --- Asp Asn Cys Asp Ser Ser Leu Ser Arg Thr Tyr Ala Ile Ser Ser Ser Val Ile Ser Phe Tyr Ile Pro Val Ala Ile
GAG ACC ATA --- GAC AAC TGT GAC TCC AGC CTC AGC AGG ACA TAT GCC ATC TCA TCC TCT GTA ATA AGC TTT TAC ATC CCT GTG GCC ATC
Asp Glu Asp Thr Arg Leu
239
Met Ile Val Thr Tyr Thr Arg Ile Tyr Arg Ile Ala Gln Lys Gln Ile Arg Arg Ile Ala Ala Leu Glu Arg Ala Ala Val His Ala Lys
ATG ATT GTC ACC TAC ACC AGG ATC TAC AGG ATT GCT CAG AAA CAA ATA CGG CGC ATT GCG GCC TTG GAG AGG GCA GCA GTC CAC GCC AAG
Ser Ser
269
Asn Cys Gln Thr Thr Thr Gly Asn Gly Lys Pro Val Glu Cys Ser Gln Pro Glu Ser Ser Phe Lys MET Ser Phe Lys Arg Glu Thr Lys
AAT TGC CAG ACC ACC ACA GGT AAT GGA AAG CCT GTC GAA TGT TCT CAA CCG GAA AGT TCT TTT AAG ATG TCC TTC AAA AGA GAA ACT AAA
Ala Asn Ala Ser
299
Val Leu Lys Thr Leu Ser Val Ile MET Gly Val Phe Val Cys Cys Trp Leu Pro Phe Phe Ile Leu Asn Cys Ile Leu Pro Phe Cys Gly
GTC CTG AAG ACT CTG TCG GTG ATC ATG GGT GTG TTT GTG TGC TGT TGG CTA CCT TTC TTC ATC TTG AAC TGC ATT TTG CCC TTC TGT GGG
Ser Met Val
329
Ser Gly Glu Thr Gln Pro Phe Cys Ile Asp Ser Asn Thr Phe Asp Val Phe Val Trp Phe Gly Trp Ala Asn Ser Ser Leu Asn Pro Ile
TCT GGG GAG ACG CAG CCC TTC TGC ATT GAT TCC AAC ACC TTT GAC GTG TTT GTG TGG TTT GGG TGG GCT AAT TCA TCC TTG AAC CCC ATC
Glu Ile
359
Ile Tyr Ala Phe Asn Ala Asp Phe Arg Lys Ala Phe Ser Thr Leu Leu Gly Cys Tyr Arg Leu Cys Pro Ala Thr Asn Asn Ala Ile Glu
ATT TAT GCC TTT AAT GCT GAT TTT CGG AAG GCA TTT TCA ACC CTC TTA GGA TGC TAC AGA CTT TGC CCT GCG ACG AAT AAT GCC ATA GAG
Gln Thr
389
Thr Val Ser Ile Asn Asn Asn Gly Ala Ala MET Phe Ser Ser His His Glu Pro Arg Gly Ser Ile Ser Lys Glu Cys Asn Leu Val Tyr
ACG GTG AGT ATC AAT AAC AAT GGG GCC GCG ATG TTT TCC AGC CAT CAT GAG CCA CGA GGC TCC ATC TCC AAG GAG TGC AAT CTG GTT TAC
Val Val Asp
419
Leu Ile Pro His Ala Val Gly Ser Ser Glu Asp Leu Lys Lys Glu Glu Ala Ala Gly Ile Ala Arg Pro Leu Glu Lys Leu Ser Pro Ala
CTG ATC CCA CAT GCT GTG GGC TCC TCT GAG GAC CTG AAA AAG GAG GAG GCA GCT GGC ATC GCC AGA CCC TTG GAG AAG CTG TCC CCA GCC
Gly Lys
446
Leu Ser Val Ile Leu Asp Tyr Asp Thr Asp Val Ser Leu Glu Lys Ile Gln Pro Ile Thr Gln Asn Gly Gln His Pro Thr
CTA TCG GTC ATA TTG GAC TAT GAC ACT GAC GTC TCT CTG GAG AAG ATC CAA CCC ATC ACA CAA AAC GGT CAG CAC CCA ACC TGAACTCGCAG
Val His Ser Ser
1349
ATGAATCCTGCCACACATGCTCATCCCAAAAGCTAGAGGAGATTGCTCTGGGGTTTGCTATTAAGAAACTAAGGTACGGTGAGACTCTGAGGTGTCAGGAGAGCCCTCTGCTGCTTTCC
1468
AACACACAATTAACTCCGTTTCCAAATACATTCCAGTGTATTTTCTGTGTTGTTCATAGTCAATCAAACAGGGACACTACAAACATGGGGAGCCATAAGGGACATGTCTTTGGCTTCAG
1587
AATTGTTTTTAGAAATTTATTCTTATCTTAGGATTTACCAAATAGGGCAAAGAATCAACAGTGAACAGCTTCACTTAAAATCAAATTTTTCTGGGAAGAAAATGAGATGGGTTGAGTTT
1706
GCTGTATACAAACAGGTGCTAACACTGTTCCCAGCAAAGTTTTCAGATTGTAAAGGTAGGTGCATGCCTTCATAAATTATTTCTAAAACATTAATTGAGGCTTACAGTAGGAGTGAGAA
1825
ATTTTTTTCCAGAATTGAGAGATGTTTTGTTGATATTGGTTCTATTTATTTATTGTATATATGGATATTTTTAATTTATGATATAATAAATATATATTTATCATATTTAATAGGATAAA
1944
TTAATGAGTTTTATCCAAGACCTTACAACCACATTTCTGGCCATTTAACTAGCACTTTATAAGCCAATGAAGCAAACACACAGACTCTGTGAGATTCTAAATGTTCATGTGTAACTTCT
2063
AGA

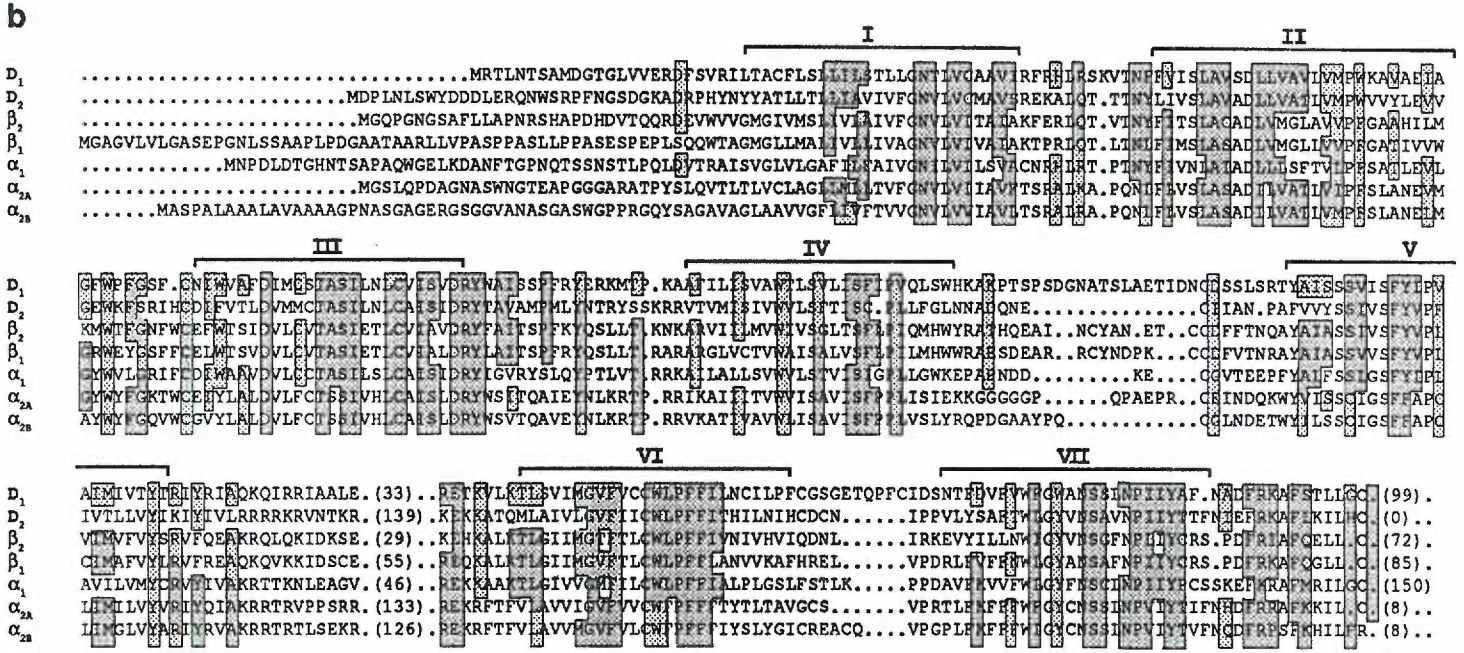

b
I
II
III
IV
V
VI
VII
D1
D2
β2
β1
α1
α2A
α2B

Fig.1 Sequences of the human and rat dopamine D_1 receptor and comparison with the sequences of other catecholamine receptors. a) Nucleotide and deduced amino-acid sequences of the human and rat D_1 dopamine receptors. Numbering begins with the first methionine of the long open reading frame and is beneath the nucleotide sequence at the right of each line. The deduced amino-acid sequence of human D_1 receptor is shown above the nucleotide sequence. For rat D_1 sequences, the coding region and their differences with human D_1 are shown below the human D_1 sequences. The double underline denotes the small open reading frame in the 5' untranslated region. The postulated N-glycosylation sites are indicated by asterisks and the putative protein kinase A phosphorylation sites have a line above them. Dotted lines denote deletion. b) Alignment of the amino-acid sequence of the human D_1, human D_2, human β_2, human β_1, hamster α_1, human α_{2A} and human α_{2B} receptors. Shaded amino acids represent residues that are conserved in at least three receptors and the D_1. The putative transmembrane domains are bracketed and labelled by Roman numerals. The number of residues in the variable third cytoplasmic loop and at the C-terminus are shown in parentheses.

Methods. Double-stranded cDNA was synthesized from rat striatum polyA$^+$ mRNA. Two degenerate oligonucleotides(III GAGTCGACCTGTG{C/T}G{C/T}{C/G}AT{C/T}{A/G}CIIT{G/T}GAC{C/A}G{C/G}TAC, VI CAGAATTCAG{T/A}AGGGCAICCAGCAGAI{G/C}{G/A}{T/C}GAA) were designed based on the relatively conserved regions of

receptor transmembrane domains III and VI. The rat striatum cDNA served as template in 30 cycles of PCR with one minute of denaturation at 95°C, two minutes of annealing at 45°C, and three minutes of extension at 72°C in 100 µl containing 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris pH 8.4, 200 µM each dNTP, and 2.5 units of Taq polymerase(Cetus). After phenol/chloroform extraction and ethanol precipitation, the PCR products were double digested with EcoRI and SalI and separated on a 1.2% agarose gel. The PCR products from 450 to 700 bp in size were extracted (GeneClean) and subcloned into M13mp18 and M13mp19. A total of 24 clones were sequenced. One clone, R213 (530 bp), was especially interesting (see text). Random-primed R213 probe was then used to screen in succession a rat striatum cDNA library in λgt10, a human genomic library in EMBL-3 and a rat genomic library both in λDASH under stringent hybridization conditions: 50% formamide, 5xSSC, 5xDenhardt's, 0.1% sodium pyrophosphate, 0.5% SDS and 100 ug/ml denatured salmon sperm DNA at 37°C. Colony Plaque Screen filters(NEN) were washed at 0.2xSSC, 0.1% SDS at 55°C and exposed overnight at -70°C to Kodak X-ray film with an intensifying screen. One positive rat cDNA clone, five rat genomic clones and eight human genomic clones were isolated. One human genomic clone, HGR213-1, was further characterized by Southern blot analysis and a 3.0 kb EcoRI/SacI fragment which hybridized to R213 was subcloned and sequenced. The rat coding sequence was obtained by sequencing of a partial rat cDNA clone and a rat genomic clone. Sequencing was done by

the Sanger dideoxy chain termination method using Sequenase (US Biochemical Corporation).

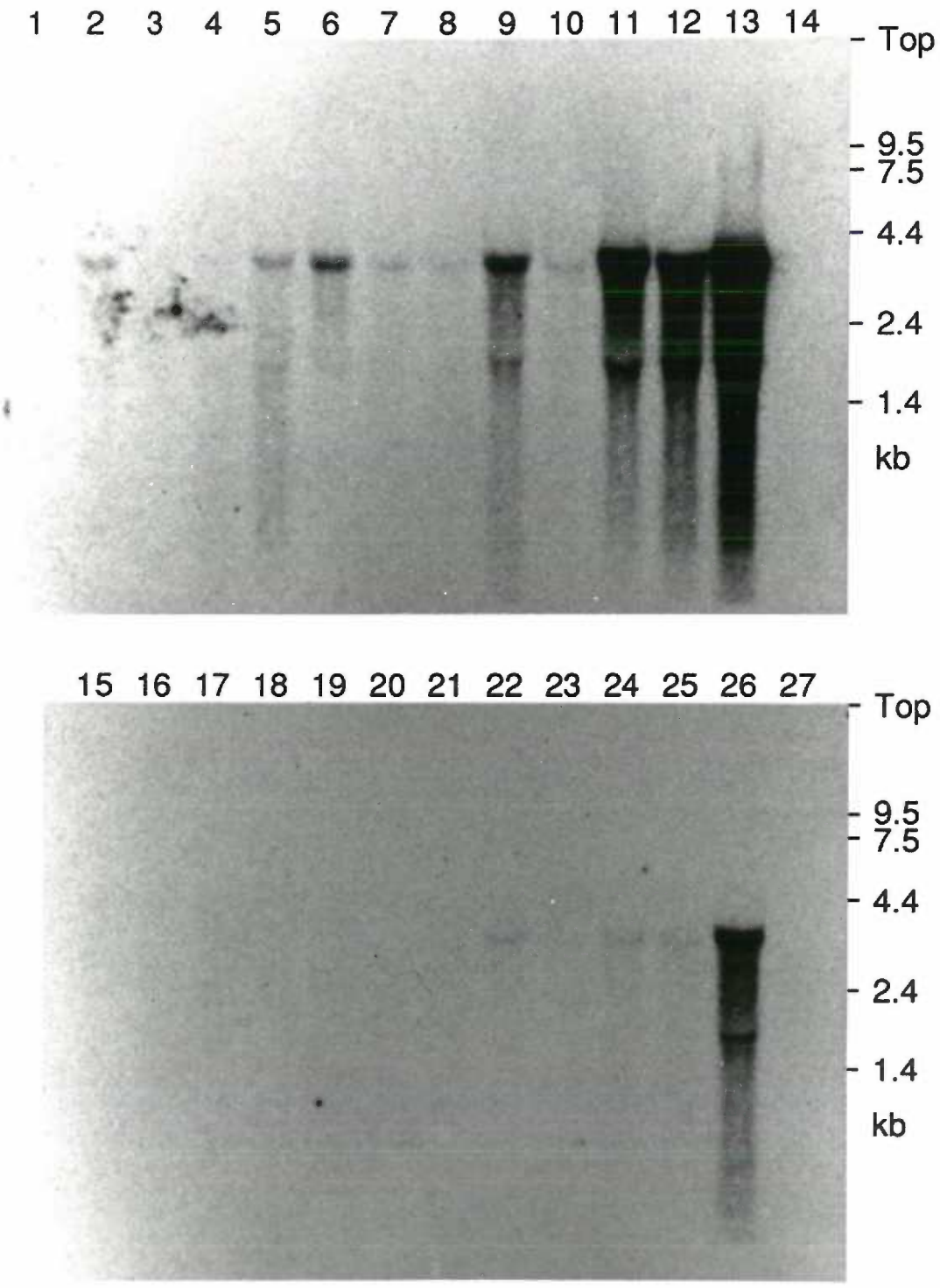

1 2 3 4 5 6 7 8 9 10 11 12 13 14
Top
9.5
7.5
4.4
2.4
1.4
kb
15 16 17 18 19 20 21 22 23 24 25 26 27
Top
9.5
7.5
4.4
2.4
1.4
kb

Fig.2 Northern blot analysis of R213 transcripts in rat brain regions, pituitary and peripheral tissues. Northern blot analysis was performed as previously described except random-primed R213 was used as hybridization probe[7]. Each lane contained 20 ug of total RNA. Numbers on the right indicate kb as determined from RNA size markers (BRL). Lane 1, olfactory bulb; 2, hippocampus; 3, cerebellum; 4, posterior cortex; 5, anterior cortex; 6, thalamus; 7, hypothalamus; 8, medulla; 9, amygdala; 10, mesencephalon; 11, septum; 12, posterior basal ganglia; 13, anterior basal ganglia; 14, neurointermediate lobe of pituitary; 15, muscle; 16, ventricle; 17, atrium; 18, lung; 19, adrenal; 20, kidney; 21, liver; 22, pineal; 23, anterior lobe of pituitary; 24, hypothalamus; 25, mesencephalon; 26, posterior basal ganglia; 27, neurointermediate lobe of pituitary.

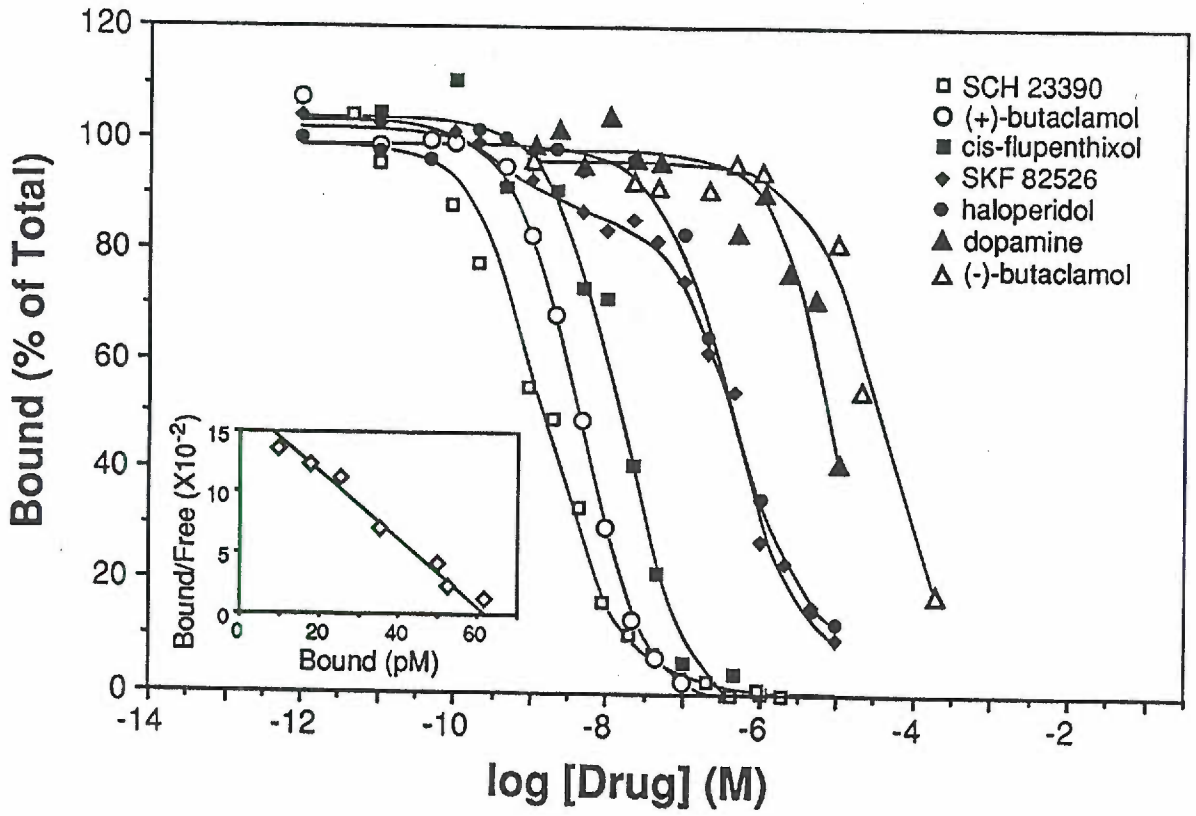

Bound (% of Total)
log [Drug] (M)
120
100
80
60
40
20
0
-14
-12
-10
-8
-6
-4
-2
SCH 23390
(+)-butaclamol
cis-flupenthixol
SKF 82526
haloperidol
dopamine
(-)-butaclamol
Bound/Free (X10-2)
15
10
5
0
0
20
40
60
Bound (pM)

Fig.3 Binding of [^{3}H]SCH23390 to membranes prepared from COS-7 cells transfected with HGR213-1. Representative curves show the competitive inhibition of [^{3}H]SCH23390 specific binding by different drugs. The inset shows a Scatchard transformation of saturation binding. The average Ki values from three independent experiments were: SCH23390 0.4 nM, (+)butaclamol 2.1 nM, cis-flupenthixol 5.6 nM, haloperidol 203 nM, dopamine 2.3 μM, (-)butaclamol 19 μM and SKF82526 0.2 nM (high affinity) and 150 nM (low affinity). In the Scatchard plot shown the Kd and Bmax values for membranes prepared from transfected COS-7 cells were 0.3 nM and 2 pmole per mg protein respectively.

Methods. The 3.0 kb EcoRI/SacI fragment of HGR213-1 was inserted between the unique HindIII and BamHI sites of eukaryotic expression vector pBC12BI[27]. A modified calcium phosphate method[28] was used for the transfection of COS-7 cells. About 45 μg plasmid DNA were used for each large 150mm plate. At 48 h after transfection, cells were rinsed with TEM buffer(25 mM Tris pH 7.4, 6 mM $MgCl_2$, 1 mM EDTA) and scraped off plates. Membranes were prepared by homogenizing cells with a ConTorque homogenizer at 4°C in TEM buffer. The homogenate was centrifuged at 800g for 10 min and the pellet was subjected to a second homogenization and centrifugation. Supernatants were pooled and centrifuged at 100,000g for 1 h. The pellet was then resuspended in TEM buffer at appropriate protein concentration and stored in small aliquots at -70°C. Binding assays were performed in duplicate in a volume of 500

ul containing 50 mM Tris pH 7.4, 0.9% NaCl, 0.025% ascorbic acid, 0.001% BSA, [^{3}H]SCH23390 (Amersham, 69 Ci mmol-1) and tested drugs. In all competition binding assays, 0.7 nM [^{3}H]SCH23390 was inhibited by various concentrations of unlabelled drugs. Binding was initiated by the addition of membrane preparation (20-30 ug protein) and carried on at 30°C for 1 h. Nonspecific binding was defined in the presence of 10 µM (+)Butaclamol. The samples were filtered through glass fiber filters (Schleicher and Schuell No.32) and washed three times with 4 ml ice-cold 10 mM Tris pH 7.4. The radioactivity retained on the filter was counted using a Beckman LS6800 scintillation counter. The 50% inhibitory concentration values (IC50) calculated from the curves were converted to Ki values as described[7]. Inhibition was fit best by assuming the existence of only one class of binding site except in the case of inhibition by the agonist SKF82526 which was best fit by assuming the presence of two classes of binding sites. A LIGAND computer program was used for data analysis and curve fitting.

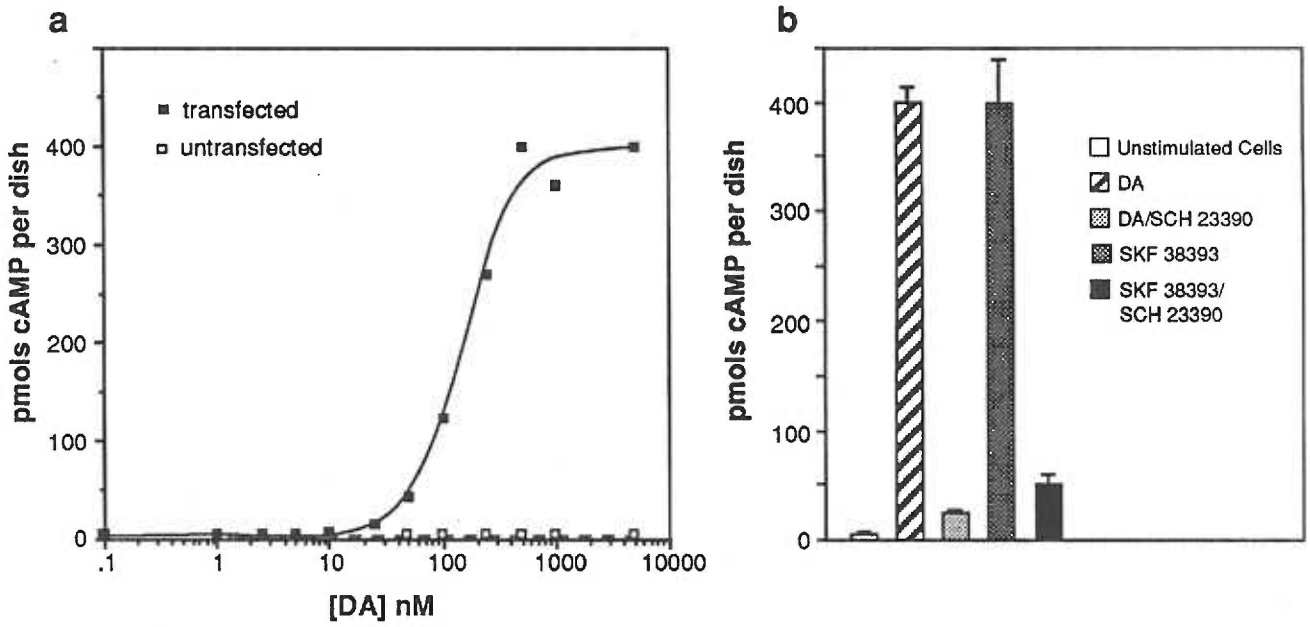

a
transfected
untransfected
pmols cAMP per dish
0
100
200
300
400
.1
1
10
100
1000
10000
[DA] nM
b
pmols cAMP per dish
0
100
200
300
400
Unstimulated Cells
DA
DA/SCH 23390
SKF 38393
SKF 38393/
SCH 23390

Fig.4 a) Dopamine-induced cAMP accumulation in human embryonic kidney 293 cells transfected with HGR213-1. Intracellular cAMP (ordinate) was measured as a function of dopamine (DA) concentration (abscissa) after transient expression of HGR213-1. Triplicate plates were analyzed for each point. The half-maximal stimulation concentration of dopamine (EC50) of the curve shown is 154 nM. b) Stimulation of cAMP accumulation in 293 cells by dopamine and SKF38393 and the antagonizing effect of SCH23390. cAMP production in 293 cells was stimulated by the agonists dopamine (125nM) and SKF38393 (250nM) and antagonized by SCH23390 (500nM). Methods. Exponential growing human 293 cells (in 60-mm dishes) were transfected with 5 μg of HGR213-1 expression plasmid DNA in PBC12BI[27] using a modified CaPO4 method[28]. The dishes were rinsed twice with DMEM plus 10% fetal calf serum after 18 h. Two days later, the plates were rinsed twice with DMEM containing 1 mg/ml BSA and 0.5 mM IBMX (3-isobutyl-1-methylxanthine). The cells were then incubated for 45 min at 37°C in the same medium containing various drug. After aspiration of the medium, cells were washed twice with ice-cold Hanks buffered saline and lysed with 1 ml of 60% ethanol. The cell debris was collected and pelleted and the supernatants were lyophilized. The resulting pellets were resuspended in water and cAMP in each sample was quantitated using an assay method (Amersham) which measures the ability of cAMP in the sample to displace [8-^{3}H] cAMP from a high affinity cAMP binding protein[30]. The obtained values are

normalized for the number of cells on a 60-mm dish (approximately 10^6 cells or 400 μg membrane protein).

REFRENCES

1. Kebabian, J.W. & Calne, D.B. *Nature* **277**, 93-96, (1979).

2. Creese,I. et al. *A Rev Neurosci* **6**, 43-71 (1983).

3. Clark, D. & White, F.J. *Synapse* **1**, P347-388 (1987).

4. Walters, J.R. et al. *Science* **236**, 719-722 (1987).

5. Waddington, J.L. *J Psychopharmac* **3**, 54-63 (1989).

6. Lankford, K. et al. *Proc Natl Acad Sci U.S.A.* **85**, 4567-4571 (1988).

7. Bunzow, J.R. et al. *Nature* **336**, 783-787 (1988).

8. Saiki, R.K. et al. *Science* **239**, 487-491 (1988).

9. Dohlman, H.G. et al. *Biochemistry* **26**, 2657-2664 (1987).

10. Libert, F. et al. *Science* **244**, 569-572 (1989).

11. Boyson, S.J. et al. *J Neurosci* **6**, 3177-3188 (1986).

12. O'Dowd, B.F. et al. *J Biol Chem* **263**, 15985-15992 (1988).

13. Emorine, L.J. et al. *Science* **245**, 1118-1121 (1989).

14. Regan, J.W. et al. *Proc Natl Acad Sci U.S.A.* **85**, 6301-6305 (1988).

15. Strader, C.D. et al. *J Biol Chem* **264**, 13572-13578 (1989).

16. Lefkowitz, R.J. et al. *Cold Spring Harbor Symp. Quant. Biol.* **53**, 507-514 (1988).

17. Jarvie, K.R. et al. *Molec Pharmac* **36**, 566-574 (1989).

18. Grandy, D.K. et al. *Proc Natl Acad Sci U.S.A.* **86**, 9762-9766 (1989).

19. Kobilka, B.K. et al. *Proc Natl Acad Sci. U.S.A.* **84**, 46-50 (1987).

20. Frielle, T. et al. *Proc Natl Acad Sci U.S.A.* **84**, 7920-7924 (1987).

21. Cotecchia, S. et al. *Proc Natl Acad Sci U.S.A.* **85**, 7159-7163 (1988).

22. Kobilka, B.K. et al. *Science* **238**, 650-656 (1987).

23. Strader, C.D. et al. *J Biol Chem* **263**, 10267-10271 (1988).

24. Venter, J.C. et al. *Biochem Pharmac* **38**, 1197-1208 (1989).

25. Kishimoto, A. et al. *J Biol Chem* **260**, 12492-12499 (1985).

26. Bouvier, M. et al. *Nature* **333**, 370-374 (1988).

27. Cullen, B.R. *Meth Enzym* **152**, 684-704 (1987).

28. Chen, C. & Okayama, H. *Molec Cell Biol* **7**, 2745-2752 (1987).

29. Niznik, H.B. et al. *Molec Pharmac* **34**, 29-36 (1990).

30. Brown, B.L. et al. *Biochem J* **171**, 561-562 (1971).

We thank Linda Robbins for help with cAMP measurements, Kim Neve for suggestions and Carol Spear and Linda Raymond for help in tissue culture. We thank Richard Goodman and Michael Forte for manuscript review and Julie Tasnady and June Shiigi for manuscript preparation. This work was supported by a National Institutes of Health grant to Olivier Civelli.

Characterization of Gene Organization and Promoter Region of the Rat Dopamine D1 Receptor Gene

Qun-Yong Zhou*@, Chuanyu Li*, and Olivier Civelli*$#

* Vollum Institute for Advanced Biomedical Research, @ Department of Biochemistry, $ Department of Cell Biology and Anatomy, Oregon Health Sciences University, Portland, Oregon 97201, USA

To whom correspondence should be addressed. Phone: 503-494-5456, Fax: 503-494-4534

Abstract

Genomic and cDNA clones encoding the rat D1 receptor were isolated and sequenced. Comparison of the D1 receptor cDNA and genomic sequences revealed that the rat D1 receptor gene is organized into two exons separated by a small intron in the 5' untranslated region of its mRNA. The transcription start site is located 864 base pairs upstream from the translational initiation site. The 5'-flanking sequences of D1 receptor gene do not contain TATA and CAAT canonical sequences, but have a high G+C content, potential CRE and GRE sequences and binding sites for transcription factors such as Sp1, Ap1 and Ap2. Transfection studies using the D1 5'-flanking sequence and CAT gene fusion constructs have demonstrated that: (1) the D1 receptor gene promoter is active in D1-expressing neuroblastoma NS20Y cells but inactive in D1-deficient glioma C6 and kidney 293 cells; (2) the information contained within 735 base pairs of 5'-flanking sequence of D1 gene appears to be sufficient to confer its cell-specific expression; (3) the D1 gene promoter responds to cAMP induction, suggesting the existence of an autoregulation mechanism by which the stimulation of D1 receptor exerts a positive feedback on its own gene expression.

Introduction

Dopamine plays an important role in motor, emotive, cognitive, and neuroendocrine functions of the brain. Disturbances of dopaminergic neurotransmission are thought to contribute to etiology and/or pathology of neurological disorders such as Parkinson's disease and psychiatric disorders such as schizophrenia (Creese, 1986). Molecular, biochemical, physiological and pharmacological studies have shown that dopamine exerts its diverse physiological functions through interaction with several dopamine receptors (Kebabian and Calne, 1979; Creese, 1986; Civelli et al., 1991). The two predominant ones are the D1 and D2 receptors. The D1 receptor is linked to the stimulation of adenylate cyclase and may also activate phospholipase C and Ca++ mobilization (Kebabian and Calne, 1979; Mahan et al., 1990). The D2 receptor is linked to the inhibition of adenylate cyclase and Ca++ channel and opening of K+ channel (Creese, 1986).

Traditionally, the behavioral effects of dopaminergic agents were attributed to D2 receptor while the roles of D1 receptor in the brain were less clear. Recent studies have suggested that D1 receptor plays important roles in motor control (Clark and White, 1987), cognition (Sawaguchi and Goldman-

Rakic, 1991), neuroendocrine (Reubi et al., 1977; Girault et al., 1986; Benkirane et al., 1987; Gerfen et al., 1990), central nervous system development (Lankford et al., 1988; Rodrigues and Dowling, 1990; Mattingly et al., 1991), and modulating the activity of D2 receptors (Clark and White, 1987; Waters et al., 1987; Waddington, 1989; Bertorello et al., 1990). In addition, D1 receptor mediates the activation of immediate-early genes by psychomotor stimulants and may be involved in nicotine addiction (Acquas et al., 1989; Graybiel et al., 1990; Young et al., 1991).

We and others have recently cloned the human and rat D1 receptor gene that is coupled to the stimulation of adenylate cyclase (Zhou et al., 1990; Dearry et al., 1990; Sunahara et al., 1990; Monsma et al., 1990). The D1 receptor gene has also been localized to long arm of human chromosome 5 at 5q35.1 (Grandy et al., 1990; Sunahara et al., 1990). The availability of D1 receptor cDNA and genomic clones have helped advance our understanding of the dopaminergic circuits of the brain (Mansour et al., 1991; Moine et al., 1991; Weiner et al., 1991; Fremeau et al., 1991), address the synergistic interaction between D1 and D2 receptors (Piomelli et al., 1991), elucidate cell-specific signal transduction of D1 receptors (Liu et al., 1992) and perform disease linkage studies (Jensen et al., 1992). In this report we describe the structure of the rat D1 receptor gene and the promoter

structure of the gene that may play a role in its expression regulation.

Materials and Methods

Materials

Restriction enzymes were purchased from Boeringer Mannheim and New England Biolabs. T4 polynucleotide kinase, T4 ligase, S1 nuclease, Klenow fragment of E. Coli DNA polI, murine reverse transcriptase and RNase inhibitor were all obtained from Bethesda Research Laboratories. The Sequenase kit was a product of United States Biochemical Corp. [^{3}H]acetyl CoA and water-immersible liquid scintillation solution Econoflour-2 were purchased from NEN/DuPont. pBASIC-CAT, pSV40-CAT, and CAT enzyme standard were obtained from Promega. 8-bromo-cAMP, dexamethasone, phorbol-12-myristate-13-acetate and chloramphenicol were from Sigma.

Isolation and characterization of D1 receptor cDNA and genomic clones: A rat striatal cDNA library in λgt10 and a rat genomic library in λDASH were screened by plaque hybridization with a partial rat D1 cDNA clone (Sambrook et al., 1989). The probe was labelled by random-priming method to specific activity of about 10^9 cpm/mg using a Prime-It kit

from Stratagene. Three cDNA clones and five genomic clones were purified. One cDNA clone with the longest cDNA insert and one genomic clone called λRGD1-2 were further characterized by restriction mapping and Southern blotting hybridization analysis (Sambrook et al., 1989). The cDNA insert and two genomic EcoRI fragments of about 2.1 and 4.1 kb, that hybridized to the partial rat D1 cDNA probe in Southern blot were subcloned into pBluescript (Stratagene) or pGemblue (Promega). Smaller fragments from the resulting pBluescript and pGemblue plasmids were subcloned into M13MP18 and M13MP19 and sequenced by the dideoxy chain termination method using the Sequenase kit. Sometimes synthetic oligonucleotides were used to faciliate DNA sequencing. Nucleotide sequence analysis and homology comparisons were done on SUN/UNIX computer system with softwares provided by IntelliGenetics Inc.

Primer extension analysis: Total RNAs were isolated from rat brain and liver tissues by guanidinium thiocyanate method (Sambrook et al., 1989). 30 μg total RNA was hybridized to $2*10^5$ cpm of end-labelled antisense oligonucleotide (5'GTTCTCAGAGCTTCCTGGGGAGAGGAACCT3') corresponding to nucleotides 751 to 781 upstream from the translation start site (Fig.2). The probe was coprecipitated with total RNAs and resuspended in 30 ml hybridization buffer (80% formamide, 30 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA), heated to 90^0C for 10 min and hybridized at 30^0C overnight. The samples were

diluted fourfold and precipitated with 2 volume ethanol, resuspended in 50 ml of 50 mM Tris pH 8.0, 75 mM KCl, 3 mM MgCl2, 100 mM DTT, 40 units/ml RNasin, 1 mM dNTPs and 200 units of murine reverse transcriptase. Elongation was carried on for 2 hrs at 37^0C. The cDNA products were extracted once with phenol/chloroform and precipitated with 2.5 volume of ethanol and analyzed in a 6% sequencing gel.

Construction of CAT plasmids: The 4.1 kb EcoRI fragment of lamda RGD1-2 was subcloned into pGemblue resulting in pGem4.1. The CAT plasmids containing different lengths of 5'-flanking sequences of the D1 gene were constructed from pGem4.1. p0.7CAT was constructed by ligating the 0.8 kb PstI/SpeI fragment(-734 to +74) of pGem4.1 into pBASIC-CAT digested with PstI and XbaI. For constructing p1.2CAT the 1.3 kb XbaI/SpeI (-1274 to +74) of pGem4.1 was inserted into XbaI site of pBASIC-CAT and the appropriate orientation of p1.2CAT was chosen by restriction analysis. Plasmid DNA was isolated by SDS-NaOH lysis method and was further purified by one cycle of CsCl gradient ultracentrifugation (Sambrook et al., 1989).

Cell Culture, DNA transfection and CAT assays: NS20Y neuroblastoma, C6 glioma and embryonic kidney 293 cells were grown to about 40% confluency in 100mm dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cells were transfected with 10 µg plasmid DNA (pBASIC-

CAT, pSV40-CAT, and D1-CAT fusion constructs po.7CAT and p1.2CAT) using the modified CaPO4 method (Chen and Okayama, 1987). Two days after transfection, cells were washed twice with TM buffer (10 mM Tris-HCl, pH 7.8, and 2 mM MgCl2), lysed with 500 ml of 0.1 M Tris-HCl, pH 7.8 and 0.1% Triton X-100 and supernatant samples were kept at -70^0C. Protein concentration in the lysates was measured by Lowry-Phenol method using bovine serum albumin as a standard. For measuring the CAT activities, lysates were incubated at 65^0C for 15 min to minimize endogenous acylation and deacylation activity and 50 µg lysate protein (in 50 µl volume) was added to a 200 µl mixture containing 0.1 M Tris-HCl, pH 7.8, 1 mM choloramphenicol, 1 µCi [^{3}H]acetyl CoA (specific activity 200 µCi/mmol) and overlied with 5 ml water-immersible scintillation solution Econoflour-2 (Neumann et al., 1987). CAT activities were monitored by continuing counting from 20 min to 4 hr after the reaction starts (setting 0.1 min per sample) and were calculated from a standard curve developed by using different dilutions of CAT enzyme standard. Positive control plasmid pSV40-CAT converted 8.0% to 10.0% of substrate [^{3}H]acetyl CoA in NS20Y, C6 and 293 cells. The basal CAT activities of untransfected NS20Y, C6 and 293 cells converted about 0.2% of substrate [^{3}H]acetyl CoA. For drug treatment, 24 hr after transfection, 8-Bromo-cAMP (8-Br-cAMP), phorbol-12-myristate-13-acetate (PMA) or dexamethasone (dex) were added to cells at a final concentration of 1 mM,

100 nM and 1 μM respectively and maintained for approximately 18 hours.

Results

Isolation and characterization of rat D1 cDNA clones: A rat striatal cDNA library was screened with a partial D1 receptor cDNA (Zhou et al., 1990). Three cDNA clones were identified. The 3.6 kb insert of one cDNA clone was entirely sequenced. An open reading frame (ORF) of 446 amino acids was identified from the nucleotide sequence (Fig.2). This ORF is 41 residues shorter compared to the other report of rat D1 receptor (Monsma et al., 1990). The correctness of our assignment of the translation start site is supported by the presence of a Kozak consensus sequence (Kozak, 1984) around the start ATG and an in-frame stop codon 120 nucleotide upstream the start codon and by the comparison of rat and human D1 sequences (Fig.2). Computer sequence comparison revealed that the coding region of rat D1 receptor cDNA is about 86% identical to the human D1 gene at nucleotide level and 92% identical at amino acid level (Zhou et al., 1990; Dearry et al., 1990; Sunahara et al., 1990). The highest sequence similarity between rat and human D1 receptor was found in the putative transmembrane domains. Eight copies of the AUUUA sequence, which is thought to mediate selective mRNA degradation (Shaw and Kamen, 1987), were found in the 3' untranslated region of the rat D1 cDNA with six copies being

clustered in a short region of 70 base pairs (Fig.2). Thus D1 receptor mRNA may have a short life span.

Isolation and characterization of rat D1 receptor genomic clones: Screening of a rat genomic library with a partial D1 cDNA probe yielded 5 positive clones, designated as RGD1-1 to RGD1-5. One of them, RGD1-2, containing a 17 kb insert was further characterized by restriction enzyme mapping and Southern blot hybridization (Sambrook et al., 1989). A schematic representation of this 17 kb rat genomic fragment displaying 5'-flanking sequence, two exons, one intron, restriction enzyme sites and relative locations of transcriptional and translational sites is shown in Fig.1. Over 6 kb of the genomic insert of RGD1-2 was sequenced (Fig.2).

Comparison of rat genomic and cDNA sequences revealed the existence of an intron in the 5' untranslated region of the D1 mRNA (Fig.2). The intron is relatively small (115 bp) and the sequences surrounding the intron/exon junctions conform to the consensus sequences for splice junctions (Mount, 1982). The first exon contains 313 base pairs of 5' untranslated region of D1 mRNA and all the coding region and the 3' untranslated region is contained in the second exon. This gene organization of D1 receptor is similar to that of the muscarinic receptors (Bonner et al., 1987).

Identification of putative transcriptional initiation site: Primer extension assays were used to identify the putative transcription initiation site of D1 receptor gene. When an end-labelled antisense oligonucleotide complementary to nucleotides 751 to 781 upstream from the translation start codon was used as a primer, one elongation product of 113 bps was detected (Fig.3). The site of putative transcriptional initiation was identified as the cytidine, 864 nucleotides upstream the translation start site.

Promoter activity of 5'-flanking region of the rat D1 receptor gene: D1-CAT fusion constructs and pSV40-CAT were introduced into NS20Y neuroblastoma, C6 glioma and embryonic kidney 293 cells by DNA transfection. In contrast to pSV40-CAT, the CAT activities of D1-CAT fusion constructs were not uniform among different cell lines. The relative CAT activities of D1-CAT fusion genes in NS20Y cells were 10 to 20 fold higher than in the glioma C6 and 293 cells. As shown in Fig.4, the D1 5'-flanking sequence (-735/+78) functioned 60% as efficiently as the SV40 promoter in NS20Y cells in which the D1 receptor is endogenously expressed (Barton and Sibley, 1990). When a longer D1 5'-flanking sequence (-1274/+78) was fused to the CAT gene, however, CAT activity was decreased about twofold in NS20Y cells, i. e. to 33% of that of pSV40-CAT construct (Fig.4). The CAT activities of D1-CAT fusion constructs p0.7CAT and p1.2CAT in C6 and 293 cells, in which D1 receptors are not expressed (Zhou et al.,

1990), were 2-5% of that of pSV40-CAT (Fig.4). The observation that p0.7CAT, which contained 735 bp 5'-flanking sequence of D1 receptor gene, was active in D1-positive NS20Y cells but inactive in D1-deficient C6 and 293 cells suggesting that information contained within the 735 base pairs of 5'-flanking sequence of D1 receptor gene appears to be sufficient to confer its cell-specific expression.

Potential regulatory sequence motifs within the D1 receptor promoter region: Analysis of the D1 receptor 5'-flanking sequence revealed several structural features typical of a housekeeping gene (Tab.1 and Fig.2). First, no TATA and CAAT canonical sequences were found within 500 base pairs of the presumed transcriptional start site defined by primer extension analysis. Second, it had a high G+C content, averaging 65% in the first 500 base pairs of 5'-flanking sequences. Third, there were three potential binding sites for the transcription factor Sp1, sequences commonly observed in promoters of housekeeping genes (Dynan, 1986; Briggs et al., 1986; Mitchell and Tjian, 1989).

The 5'-flanking sequence of the D1 receptor gene also contained sequences representing other putative cis-elements (Tab.1, Fig.2). Among these are multiple potential binding sites for transcription factor Ap2 (Becker et al., 1987) and one possible binding site for Ap1 (Lee et al., 1987). An 8-bp palindromic sequence (TGGCGCCA) that is quite similar to

consensus cAMP response element (TGACGTCA) was found 20 bp upstream from the transcription initiation site (Goodman, 1990). A potential glucocorticoid response element (GRE) was also noticed at 299-313 upstream the transcription start site (Tsai et al., 1988). The existence of multiple putative transcription factor binding sites suggests that several DNA binding proteins may work in conjunction with each other to mediate the level of D1 receptor gene transcription.

To test whether the D1 5'-flanking sequence is responsive to cAMP, phorbol ester and glucocorticoids, 8-Br-cAMP, PMA, and dexamethasone were added to NS20Y cells transfected with p0.7CAT. As shown in Fig.5, 8-Br-cAMP increased the CAT activity of p0.7CAT to twofold whereas PMA and dexamethasone had nearly no effect. These results suggested that 5'-flanking sequences of the rat D1 receptor gene can be responsive to cAMP. Although PMA and dexamethasone themselves had little effect on CAT activity of p0.7CAT, both of them appeared to be able to potentiate the action of 8-Br-cAMP (Fig.5).

Discussion

We and others have recently reported the cloning and functional characterization of dopamine D1 receptor gene and cDNA (Zhou et al., 1990; Dearry et al., 1990; Sunahara et al., 1990; Monsma et al., 1990; Liu et al., 1992). Partial

characterization indicated that D1 receptor gene has no introns in the coding region (Zhou et al., 1990). In this report, we have examined in detail the gene organization of the rat D1 receptor gene, identified its transcriptional initiation site and characterized its promoter region.

Comparison of cDNA and genomic sequence indicated that the rat D1 receptor gene is organized into two exons with a small intron interrupting the 5' untranslated region of the mRNA (Fig.1). Within the G-protein coupled receptor family, introns in the 5' untranslated regions have also been identified in genes of muscarinic receptor family and dopamine D2 receptor (Bonner et al., 1987; O'Malley et al., 1990). Primer extension studies have identified the putative transcriptional initiation site of the rat D1 receptor gene to be a cytidine, located 864 base pairs upstream the translation start site (Fig.2, Fig.3). Analysis of 5'-flanking sequence of the rat D1 receptor gene revealed several structural features typical of a housekeeping gene including no TATA and CAAT canonical boxes, a high G+C content and multiple binding sites for the transcription factor Sp1 (Dynan, 1986; Briggs et al., 1986; Mitchell and Tjian, 1989). Noteworthy, the promoter of the nerve growth factor receptor has similar structural features (Sehgal et al., 1988). The 5'-flanking region of D1 receptor gene also contained sequences that might interact with transcription factors to modulate D1 gene expression. Among these are four

potential Ap2 binding sites, one Ap1 site, one GRE-like and one CRE-like sequence that match well with their respective consensus sequences (Tab.1 and Fig.2).

Information contained within the 735 base pairs of 5'-flanking sequence of the rat D1 gene appears to be sufficient to confer its cell-specific expression. This is demonstrated by transfection studies with the D1 gene 5'-flanking sequence and CAT gene fusion constructs (p0.7CAT and p1.2CAT) showing CAT genes were active in neuroblastoma NS20Y cells that express D1 receptor endogenously (Barton and Sibley, 1990) but inactive in C6 and 293 cells that do not express D1 receptor (Fig.4).

We have shown that 8-Br-cAMP could stimulate the CAT activity of transient expression of p0.7CAT reporter plasmid in NS20Y cells and the stimulatory effect of 8-Br-cAMP on the CAT activity was potentiated by PMA and dexamethasone although they alone had almost no effect (Fig.5). An 8-bp palindromic CRE-like sequence (TGGCGCCA) is present in the rat D1 receptor gene 5'-flanking sequence. This sequence is located 20 base pairs upstream from the presumed transcriptional initiation site in agreement with the discovery that most CRE elements are within the 150 base pairs of the transcription start site (Goodman, 1990). Therefore the inducible effect of 8-Br-cAMP might be through this CRE-like enhancer element.

These data indicate that cAMP may exert positive regulation on D1 receptor gene expression.

cAMP-dependent protein kinases can enhance the transcription activities of CRE-containing genes by phosphorylating the CRE binding proteins (Montiminy et al., 1990). A positive autoregulation mechanism by which a receptor stimulatory for adenylate cyclase can exert positive feedback regulation on its own gene transcription has been observed for β2-adrenergic receptor and has been implicated for the substance P receptor (Collins et al., 1989; Collins et al., 1990; Hershey et al., 1991). Our data indicate that such positive feedback mechanism may also exist for D1 dopamine receptor (Fig.5). Activation of the D1 receptor by dopamine increases intracellular cAMP level. This cAMP elevation will not only mediate D1 receptor physiological actions and lead to D1 receptor desensitization but may also enhance D1 receptor gene transcription. Since the D1 receptor is strongly desensitized when exposed to dopamine and since protein degradation is an important factor in D1 receptor desensitization (Memo et al., 1982; Barton and Sibley, 1990), D1 receptor gene transcription and *de novo* protein synthesis appear to be necessary for D1 receptor resensitization. This compensatory positive feedback mechanism may be important for sustained activation of stimulatory receptors.

Acknowledgments: We thank Dr. J. Douglass for critical comments on the manuscript. The work was supported by NIH grants MH45614 and MH48728 to O. C.

Table I DNA sequence motifs with possible regulatory function in the rat D1 receptor gene expression

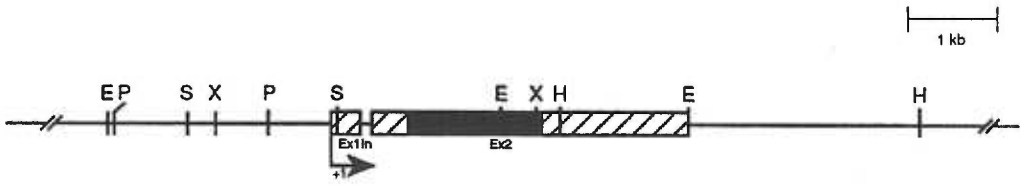
elements[a]	Consensus[b]	Sequences[c]	Positions[d]	References
Sp1	GGCGGG,CCCGCC	GGCGGG,CCCGCC	112-116,176-180,585-590	Briggs et al
Ap2	GGGGTGGGG	GGGGTGGGC,GGCGTGGGG	115-123,406-414	Becker et al
	GGGGTGGGG	GGCGTGGGC	412-420	
	CCCCACCCC	CCACACCCC,GCCCACCAC	147-155,223-231	
CRE	TGACGTCA	TGGCGCCA	20-27	Goodman
AP1	TGAGTCA	TGAACCA	197-206	Lee et al
GRE	**TG**TACANNN**TG**TTCT	**TG**TCCCTCGGGTTCT	299-313	Tsai et al
	ACATGTNNN**AC**AAGA	**AC**AGGGAGCCCAAGA		

a: CRE :cAMP response element, GRE: glucocorticoid response element
Ap1: activator protein 1, Ap2: activator protein 2,
Sp1: stimulatory protein 1

b: N stands for any nucleotide. Bold Gs are crucial for response to glucocorticoids (Tsai et al., 1988). Both strands for GRE consensus sequence are shown.

c: source of sequences fig.2 of this paper

d: negative position number according to fig.2


1 kb
E
P
S
X
P
S
E
X
H
E
H
Ex1In
Ex2
+1

Figures and Legends

Fig.1. Physical map of the genomic clone lamda RGD1-2. Exons are indicated by Ex1 and Ex2. Untranslated regions are shown by stipped areas and coding region by a filled area. The intron is marked by an In. The arrow indicates the putative transcription start site. Abbreviations for restriction sites: E: EcoRI, H: HindIII, P: PstI, Pv: PvuII, S: SpeI, X: XbaI.

```
AATTCTTTT GCTGGGGACT GGCACTGCAG GCAAAGCCAT GCCACCTGAG TCAGCCTTCG TATGGCTGGC ATCCTACTTT  -2327
AAGAGCAGT GGCCATTGCC CAGAGGCTTG GGGACACCTC TCCAAAGCAA AAGTCACTAG GGGAGTTTCA GTCCTCAAAT  -2247
GCCCTCACG GTCACCCCCA GGTTCCGTGG CTACAATCCT AAGTGCTACT GTATTGTTGC CTCTTTTAAT TTGTGTTTGC  -2167
GGTGAAAAA TGAAGAAGTA TGGTGAAATA AAGCTCCAAT CGCTTAGAGA GCAACTGCCA CACTGTGTGT GTGTGTGTAT  -2087
TGTGTGTAT GTGTGTGTGT GTGTGTGTGT GGTGAGAATC CCCTCAGGTT TTACACTGAA CTGACTCTAA AGCAGATGTT  -2007
TCAACTTGT GAATCTTGAC CCTTTGGTGG CTGGGGGGTG GGCTCATATC AGATAGTCCG TCACACATTT ACATTATAAT  -1927
CATAACAGT AACAAAATTA GTTATGGGGC AGCAACAAAA TAACTTTATG GTTGGGGGTT CTCCGCCTGT GGAACTGTAT  -1847
AAAGGGTCA CAGCATTGGG GAAGTTGAGG ACCTCTGTTC TTAAGGCATG GGTTATATGT CTGATCACGA GTCTGTGGAA  -1767
TTTGTGTGT GACAGCAAGG CTGTCAGTGG GAGGGCAGAA CTGGGCGAAC GGGCGGAGGA GCATCTGTGT CAATCATCTG  -1687
CTAGTACAC CCTTTTCCGA GATCTGATTT CTGCTGGCTA ATTAGGAGAG CTTCTTAGGC TATTTAGAGA AAATTCGAGC  -1607
TTCAGTGCG GTGTATGGTT CGCATGGAAA CAAAACTACT ATTTTATTTT TTATTTTAAA AACTGGTCCT GGAAGAAATT  -1527
CTGGCCACC AAGGGCTGAC TGAGTCCATA TCTGGTGCCC GAGTCTTAAA TAAATATCTC ATTTAATTCT TCTTAAGAAG  -1447
TCTTATCAG TAAGCACGGG AGTCCAGGCA AAGAGGTTCA CAAGTTCCAT CCTCAAGCTC TTTTCACAGG GATGGGAAGA  -1367
GGTGTTGTG TCTATGCTCT AAACGTTCCC GAGGCCACAC ATTCCTGTCT CTACTTTACC CCCGTGGTAA CTGTTTAGAT  -1287
GCGGGCTCC GTTCTAGACA TGGTCGTACA AGCGACAGTC AGACAGACAG GTCACCATCC TCATTAGCTA CTGTGTTAGC  -1207
TTGAGTAGA TTTTTCTGAG CATCATTTTC CACTCAGAGA AGTAGAGCCG TGCAAGCCTG TAAGGAAGGG TTTTGCAAGA  -1127
GTCGAAGGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTAT GCGCGTGCGT GCGCGCTTCG GGGACACAGA  -1047
CCTGCTGAG TTTATGCCAG GTCTCTGTTC ACTTGCACAG TAAGTTGACC TGTTGTCCCC ACTGCTGCTC CAGCCTTGGC   - 967
CACAGGGGA CACAAAAGGA AAATCCTGGC TTAACGAATA AAATGCTAGC CAAAGGGACT TCCTACTCTT ATGAGTTTTT   - 887
GAAGTATTT CAAGCGAGGG AGCTCAGAGA GCGGAAGACC CGCCTCTAAT CATCCAGATT CTGGCAAGCA GGGACAGGGA   - 807
AAAACAGGG TCGAGGCCCT GTACTTTGGG GTATACACTC ACTGGTTGAG GCTTCTGTGA TCTGAACCTG CAGGGGCGCA   - 727
ACGGGGAGC AGGAAACCAC AGGCACCAGC AGAGGGCGTC GGGTACCTGC TGAGCGTGCG GCGCCGTGTT TGGGGTGCTT   - 647
TGGGGCGCG GTCACGGGC TTCACTCGTG AGTTGACCGC AGAAGCGCCC TGGCGGGCGG AGTGCAGAGC GCAGAGCAAG   - 567
                                                      Sp1
GGCCTGGGA GCTGGCGCAG GGTTGGCAGA ATCCAGGAGC GTGGCCTCCC AGATCGGTGA CCACTCTCCA GCCGGGGCTC   - 487

CCGTACCCC TGTTGCGGGG CACTCAGAGA GAGCGCAGCG ATGCGGGCAG TGTCTTGGGT TAGCAGGGCG TGGGCGTGGG   - 407
                                                                          Ap2     Ap2
AGGGTCGGC TCTGATTCCG AGCTTTGGGT GGAACTTGAG GTTGGCCTGA AGAGACGCTG AGTTTTGGTT TACTTGATTT   - 327

AGCATCAGG ATTTGTCCCT CGGGTTCTGT CTTGTGCTGG GGTCCCCTGG GGGGCTGAGG TAGCCAGAGA GGGCACGGAG   - 247
                GRE
TTTGGTAGG CGTCCCACAC CCCTGCTCCA GCTGTGCCTT CAGTGAACCA TCGTGTCGTG TCGCCCGCCA CTCTGCCTGT   - 167
          Ap2                                 Ap1                 Sp1
AAGCTCAGC CCACCACCAG GGCCGGGAGG GGACGCGGAG GCGGGGGTGGG CTGTGCCCTG CTGGAACCCA GCCGGCCGGT   -  87
           Ap2                                Sp1  Ap2
CCCTCGCCC AAGCTGCTGT GCTTGCCTGG AGCGCCTGCC ACTGCTAACA GGGAGAGGGT GGCGCCACGG GGAGGCTCAG   -   7
                                                                  CRE
GTCCTGCCC TAAGAACGAG GAACCAAAGT GGGACCCAGC GCTGGGCTCC CTCAAACAGG ACAGAAAGCT GCCCCAGTGA      73
     +1
TAGTCCTGG AGGTTCCTCT CCCCAGGAAG CTCTGAGAAC CCTCCCCGGG AGAGGGGACA GCAATCTGTA GGTGGGCAAG     153
TAGCAGGAA GGGTACCGCT TCCCTGGATG CCTGGTCTGG GATTCCTTCC CCAAATCCAT CCCAGAGATT TTTCTGCATC     233
GGAGGGGAA CAGTTGCTAT GCTGACTGGG CTGACTATGG GAGCTCCAGG GGTTCTGGGA GAAGTGACCC TAAAGCAAGg    313
aggtggtct tgatggactt gtccaggaga tgatgcgctg gggttgtgtg tatctaaata tgcggtgtgc acagagtctt    393
gtgaacgac gttgtctttc cctttgcttt ttagGGCATT TGGAGAGATG CGTGCCAGGG GCTTGGAGGA GAAATGCATG     473
ATTTTAGGC CGTGTCTCAG AAAAAGAGGC AGCATCCCTG AAAAGTGACT AGAATTGACC TGGAAGAGGC CATGGACTCA     553
AGTGTGCTT AAAAGCCAAT GCTCTCCTTG GGGAATGTAG GGACCAGCCG ATGTCACAGG GACACACTGT CACAGGGACA     633
TGACCTGGA GCACCAAGCC CAGAAGACAG ATGGGAAGCA GGAGAGTCTT TACCCCGGCA TGGCTTGGAT TGCTACGGGG     713
AGCTCCTGA TGGAACCCTA CCATCCTTTA GTCCAGGCAG CAACTGGGGC TGAACAAGAA GGGGCTGGGT GGTGAGTGGT     793
GGGGGAAGT CTGGCTAAGC CTGGTCAAGA ACTTGAGGGG CAAGTCCCCG GAAGTGTGTT CCTTCTGGAA G  ATG GCT CCT
                                                                                MET Ala Pro   3

AC ACT TCT ACC ATG GAT GAG GCC GGG CTG CCA GCG GAG AGG GAT TTC TCC TTT CGC ATC CTC ACG GCC
an Thr Ser Thr MET Asp Glu Ala Gly Leu Pro Ala Glu Arg Asp Phe Ser Phe Arg Ile Leu Thr Ala  26

GT TTC CTG TCA CTG CTC ATC CTG TCC ACT CTC CTG GGC AAT ACC CTT GTC TGT GCG GCC GTC ATC CGG
rs Phe Leu Ser Leu Leu Ile Leu Ser Thr Leu Leu Gly Asn Thr Leu Val Cys Ala Ala Val Ile Arg  49

'T CGA CAC CTG AGG TCC AAG GTG ACC AAC TTC TTT GTC ATC TCT TTA GCT GTG TCA GAT CTC TTG GTG
le Arg His Leu Arg Ser Lys Val Thr Asn Phe Phe Val Ile Ser Leu Ala Val Ser Asp Leu Leu Val  72

:T GTC CTG GTC ATG CCC TGG AAA GCT GTG GCC GAG ATT GCT GGC TTT TGG CCC TTT GGG TCC TTT TGT
a Val Leu Val MET Pro Trp Lys Ala Val Ala Glu Ile Ala Gly Phe Trp Pro Phe Gly Ser Phe Cys  95

.C ATC TGG GTA GCC TTT GAC ATC ATG TGC TCT ACG GCG TCC ATT CTG AAC CTC TGC GTG ATC AGC GTG
n Ile Trp Val Ala Phe Asp Ile MET Cys Ser Thr Ala Ser Ile Leu Asn Leu Cys Val Ile Ser Val 118
```

GAC AGG TAC TGG GCT ATC TCC AGC CCT TTC CAG TAT GAG AGG AAG ATG ACC CCC AAA GCA GCC TTC ATC
Asp Arg Tyr Trp Ala Ile Ser Ser Pro Phe Gln Tyr Glu Arg Lys MET Thr Pro Lys Ala Ala Phe Ile 141

CTG ATT AGC GTA GCA TGG ACT CTG TCT GTC CTT ATA TCC TTC ATC CCA GTA CAG CTA AGC TGG CAC AAG
Leu Ile Ser Val Ala Trp Thr Leu Ser Val Leu Ile Ser Phe Ile Pro Val Gln Leu Ser Trp His Lys 164

GCA AAG CCC ACA TGG CCC TTG GAT GGC AAT TTT ACC TCC CTG GAG GAC ACC GAG GAT GAC AAC TGT GAC
Ala Lys Pro Thr Trp Pro Leu Asp Gly Asn Phe Thr Ser Leu Glu Asp Thr Glu Asp Asp Asn Cys Asp 187

ACA AGG TTG AGC AGG ACG TAT GCC ATT TCA TCG TCC CTC ATC AGC TTT TAC ATC CCC GTA GCC ATT ATG
Thr Arg Leu Ser Arg Thr Tyr Ala Ile Ser Ser Ser Leu Ile Ser Phe Tyr Ile Pro Val Ala Ile MET 210

ATC GTC ACC TAC ACC AGT ATC TAC AGG ATT GCC CAG AAG CAA ATC CGG CGC ATC TCA GCC TTG GAG AGG
Ile Val Thr Tyr Thr Ser Ile Tyr Arg Ile Ala Gln Lys Gln Ile Arg Arg Ile Ser Ala Leu Glu Arg 233

GCA GCA GTC CAT GCC AAG AAT TGC CAG ACC ACC GCA GGT AAC GGG AAC CCC GTC GAA TGC GCC CAG TCT
Ala Ala Val His Ala Lys Asn Cys Gln Thr Thr Ala Gly Asn Gly Asn Pro Val Glu Cys Ala Gln Ser 256

GAA AGT TCC TTT AAG ATG TCC TTC AAG AGG GAG ACG AAA GTT CTA AAG ACG CTG TCT GTG ATC ATG GGG
Glu Ser Ser Phe Lys MET Ser Phe Lys Arg Glu Thr Lys Val Leu Lys Thr Leu Ser Val Ile MET Gly 279

GTG TTT GTG TGC TGC TGG CTC CCT TTC TTC ATC TCG AAC TGT ATG GTG CCC TTC TGT GGC TCT GAG GAG
Val Phe Val Cys Cys Trp Leu Pro Phe Phe Ile Ser Asn Cys MET Val Pro Phe Cys Gly Ser Glu Glu 302

ACC CAG CCA TTC TGC ATC GAT TCC ATC ACC TTC GAT GTG TTT GTG TGG TTT GGG TGG GCG AAT TCT TCC
Thr Gln Pro Phe Cys Ile Asp Ser Ile Thr Phe Asp Val Phe Val Trp Phe Gly Trp Ala Asn Ser Ser 325

CTG AAC CCC ATT ATT TAT GCT TTT AAT GCT GAC TTC CAG AAG GCG TTC TCA ACC CTC TTA GGA TGC TAC
Leu Asn Pro Ile Ile Tyr Ala Phe Asn Ala Asp Phe Gln Lys Ala Phe Ser Thr Leu Leu Gly Cys Tyr 348

AGA CTC TGC CCT ACT ACG AAT AAT GCC ATA GAG ACG GTG AGC ATT AAC AAC AAT GGG GCT GTG GTG TTT
Arg Leu Cys Pro Thr Thr Asn Asn Ala Ile Glu Thr Val Ser Ile Asn Asn Asn Gly Ala Val Val Phe 371

TCC AGC CAC CAT GAG CCC CGA GGC TCC ATC TCC AAG GAC TGT AAT CTG GTT TAC CTG ATC CCT CAT GCC
Ser Ser His His Glu Pro Arg Gly Ser Ile Ser Lys Asp Cys Asn Leu Val Tyr Leu Ile Pro His Ala 394

CTG GGC TCC TCT GAG GAC CTG AAG AAG GAA GAG GCT GGT GGA ATA GCT AAG CCA CTG GAG AAG CTG TCC
Val Gly Ser Ser Glu Asp Leu Lys Lys Glu Glu Ala Gly Gly Ile Ala Lys Pro Leu Glu Lys Leu Ser 417

TCA GCC TTA TCG GTC ATA TTG GAC TAT GAC ACC GAT GTC TCT CTA GAA AAG ATC CAA CCT GTC ACA CAC
Pro Ala Leu Ser Val Ile Leu Asp Tyr Asp Thr Asp Val Ser Leu Glu Lys Ile Gln Pro Val Thr His 440

AGT GGA CAG CAT TCC ACT TGAATATTGG GTCCTCATCT CTGAGGCCAC GAGTTCCCTT GGGCTTGCTG TTAAGGAATT
Ser Gly Gln His Ser Thr 446

ACAGGAGAT CCCTCTGCTG CTTTTGGACA ATTACGAAGC TTCTCAAACT CACTGATTCC AGTGTATTCT CTAGCTTCAA 2342
GGAAATGAC TTCGGCTCTG AAATCAGTTT GGGAGTATTA TCTTAGGACA TTATAAAACA ACAACAAACA AACAAACAAA 2422
AAACAAATA GGCCAAGAGT CAACTGTAAA CAGCTTCACT TAAAAATCGA ACTTTCCAGA AAGGAAGGGT AGGAGTTGAG 2502
TTGCTGTCC AAACAGGTGC TAAAACTGTC CGAGCAGTTT TCAGATTGGA AAGGTAGGTG CATGCCTTTG TTAATTAACT 2582
CTCCAATAA TAATTGAGCC TTACAGCAGG AGTGGGATTC CTTTTTCTCA GAATTGACAG ATGCATTGTT GATGACGGTT 2662
TATTTATTT ATTTATTGTA CTATATGAAT ATTTTAAATT TATCATAGTG AATCTATATT TAACATATTT AACAGAGCAA 2742
CCAATGTGT TATCTGAGAC TGACCTCTCC ATTTGTACTA GCACTTTATG AGCCAATGAA ACATACGCGT AGACTCTGAG 2822
TTCTGAATT GTGAGTTACT TCTGGGAACA CAGCAAAGAC TGATGTGGTG GCTCCTTAAC TCGACAAGGA CACAAAGAAA 2902
GCAAGAGGA GAAGTGACTA ATGCCACCAA TGCTCCCCCT AAAAAGATTT TGAAAAGATT AGTTTTTTTT TTTTTTAAA 2982
GAAGCTACT ATTGTGTTCT GAGTGTTTTA AATGGCAGAG GCTTTCCCCG GGGCGAATTT GCACTTCTGT AAATATCTAT 3062
TAAGAACCA GCTCAAGAGG AATACAACTT TATATTTCCG CTTTTGGATG GCGAGGAAGA GCATATGCCA CTTTGTATTT 3142
TGTAAACTA ATTGGCCCTC CTTGTCATTT CTCATTTCAT GCTTGAAATA GCTTTCTGAA ACAAACAAAT GACTGTCCAG 3222
CTGGAGATC TGCAGGGTGG AGAATGAGTT GTAAATTCAC AGGTCACAGC AGCCCCTCCG ATAGCTGGGC TCATCATTGG 3302
CCTTTATCT GCCCAGGTCT AACCAAGTCG GCTGCTTAAG GGGCTACTTT TGTAGTGCTT TAATCCGAAT TTAGTATCCT 3382
TCTTTTAAA AAAAAAAGCT CTTTAATGTT AGTGGTAAAC TAGCTAATGA ACGGTACCTC ATCGCTGCAT AATACACTTC 3462
GTTGGTGGG GGCGTAGACG AGCCCTTCCC GGTGCGAGCA CCACAAAGCC ATCTGCATAG CTAGTCACAA ATGCTGTTTT 3542
CTTTCTCTG TGGGTTTGAA TCTAGTTTCC TTGTTATCAT AGCCTGGACT GCAAAAAGAT CCATCCAGTC CCCTCTTGTG 3622
GGGCATTGC AACAGTGTTT CTTTTTGTTT TTGTTTTGTT TTTGAAATGT TTACAAGGTG TTCTTTGGAA GCAGTTGCAA 3702
ACGTGGATG GAACTGAAGA AAAGGCTGAC TGGCTTGCTA ACGGTATCTC CTGCAGGGGG TTTGTACTGC GGACTTTGAA 3782
GTTTTCTCA GCTCTAAGGC TTGTATGCTT TCTTACATAC **AATAAA**CTTA TTTTGTGAAT TC 3844

Fig.2. Nucleotide sequence of the rat D1 receptor gene. The 6241 bps of the rat D1 receptor is shown. The transcriptional initiation site obtained by primer extension experiments was assigned as +1 and indicated by a bold cytidine. The 5'-flanking region and exons are shown in capital letters while the intron sequences are shown in italic lowercase letters. The 30-mer oligonucleotide sequence utilized by primer extension is shown in italics. The deduced amino acid sequence is shown below the nucleotide sequence. The mRNA instability consensus sequence AUUUA signals are underlined and a possible polyadenylylation signal AAUAAA is shown in bold. Putative regulation cis-elements (Ap1,Ap2,Sp1,CRE,GRE) are indicated by lines below the sequences.

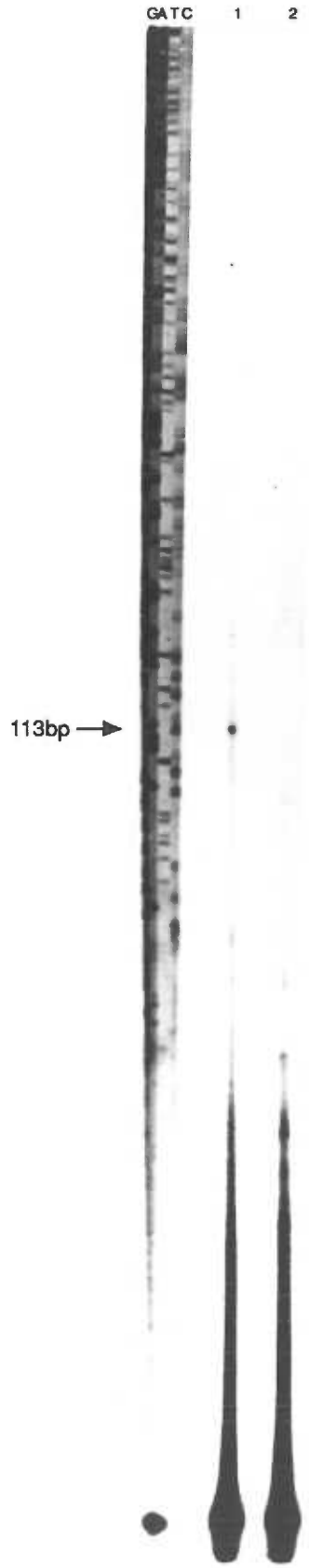

GATC
1
2
113bp

Fig.3. Identification of the rat D1 receptor gene transcriptional start site. Shown is the primer extension result of the rat D1 mRNA from the rat brain (Lane1) and the rat liver (Lane2) utilizing the 30-mer antisense oligonucleotides indicated in Fig.2. One band of 113 bp was observed in lane of rat brain RNA.

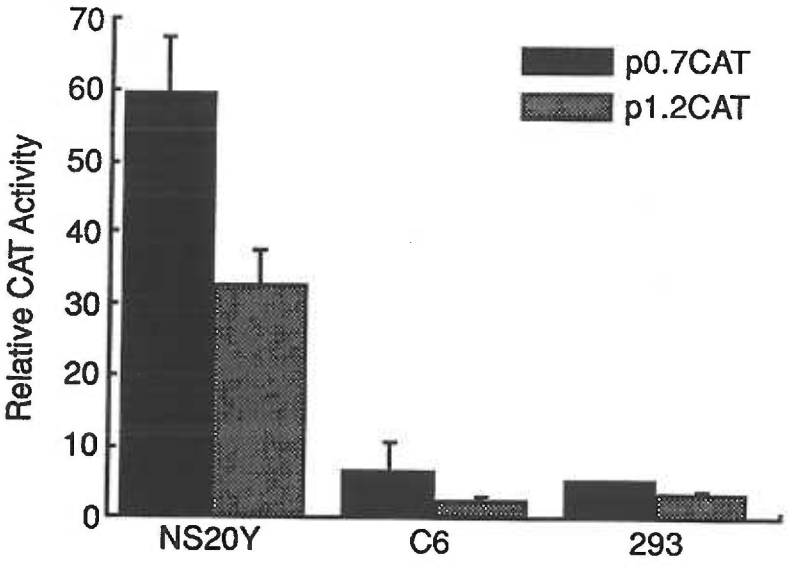

70
60
50
40
30
20
10
0
Relative CAT Activity
p0.7CAT
p1.2CAT
NS20Y
C6
293

Fig.4. Promoter activity of the rat D1 receptor gene 5'-flanking region and cell-specific expression of D1-CAT fusion genes. The CAT activities of D1-CAT fusion genes were shown as percentage of the CAT activity of pSV40-CAT in that cell line. Thin bars indicate standard error of the mean. Three to four independent experiments were done with each cell line.

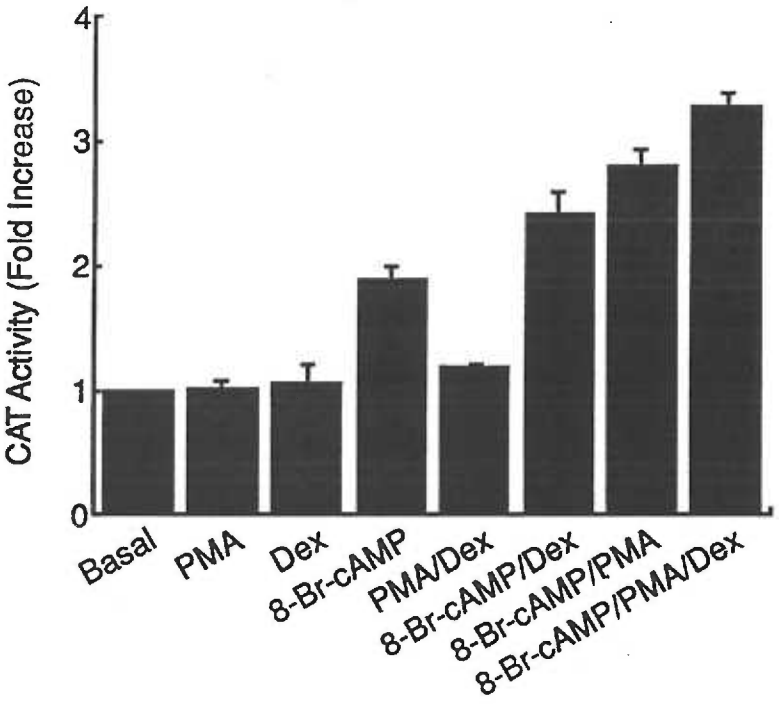

CAT Activity (Fold Increase)
0
1
2
3
4
Basal
PMA
Dex
8-Br-cAMP
PMA/Dex
8-Br-cAMP/Dex
8-Br-cAMP/PMA
8-Br-cAMP/PMA/Dex

Fig.5. Effects of 8-Br-cAMP, PMA, dexamethasone and their combinations on the CAT activities of p0.7CAT transfected into NS20Y cells. Rat neuroblastoma NS20Y cells were transfected with 10 μg of p0.7CAT reporter plasmid, followed by treatment with 8-Br-cAMP (1 mM), PMA (100 nM), dexamethasone (1 μM) and their combinations. CAT activity of p0.7CAT in transfected but drug-untreated NS20Y cells was normalized to be 1.0.

References

Acquas, E., Carboni, E., Leone, P. & DiChiara, G. (1989) SCH23390 blocks drug-conditioned place-preference and place-aversion: anhedonia (lack of reward) or apathy (lack of motivation) after dopaminergic blockage? *Psychopharmacol.* **99**, 151-155.

Barton, A. C. & Sibley, D. R. (1990) Agonist-induced desensitization of D1 dopamine receptors linked to adenylyl cyclase activity in cultured NS20Y neuroblastoma cells. *Molec. Pharmacol.* **38**, 531-541.

Becker, P. B., Ruppert, S. & Scholtz, G. (1987) Genomic footprinting reveals cell type-specific DNA binding of ubiquitous factors. *Cell* **51**, 435-443.

Benkirane, S., Arbilla, S. & Langer, S. (1987) A functional response to D1 dopamine receptor stimulation in the central nervous system: inhibition of the release of [3H]serotonin from the rat substantia nigra. *Naunyn-Schmiedebergs Arcg. Pharmacol.* **335**, 502-507.

Bertorello, A. M., Hopfield, J. F., Aperia, A. & Greengard, P. (1990) Inhibition by dopamine of (Na+-K+)ATPase activity in neostriatal neurons through D1 and D2 dopamine receptor synergism. *Nature* **347**, 386-388.

Bonner, T. I., Buckley, N. J., Young, A. C. & Brann, M. R. (1987) Identification of a family of muscarinic receptor genes. *Science* **237**, 527-532.

Briggs, M. R., Kadonaja, J. T., Bell, S. P. & Tjian, R. (1986) Purification and biochemical characterization of the promoter-specific transcription factor, Sp1. *Science* **234**, 47-52.

Chen, C & Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Molec. Cell. Biol. 7, 2745-2752.

Civelli O., Bunzow, J. R., Grandy, D. K., Zhou. Q.-Y. & Van Tol, H. H. M. (1991) Molecular biology of the dopamine receptors. *Euro. J. Pharmacol.-Molec. Pharmacol. Sec.* **207**, 277-286.

Clark, D. & White, F. J. (1987) Review: D1 receptor- the search for a function: a critical evaluation of the D1/D2 dopamine receptor classification and its functional implications. *Synapse* **1**, 347-388.

Collins, S., Bouvier, M., Bolanowski, M. A., Caron, M. G. & Lefkowitz, R. J. (1989) cAMP stimulates transcription of the β2-adrenergic receptor gene in response to short-term agonist exposure. *Proc. Natl. Acad. Sci. USA* **86**, 4853-4857.

Collins, S., Altschmied, J., Herbsman, O., Caron, M. G., Mellon, D. L. & Lefkowitz, R. J. (1990) A cAMP response element in the β2-adrenergic receptor gene confers transcriptional autoregulation by cAMP. *J. Biol. Chem.* **265**, 19330-19335.

Creese, I. (1986) Dopamine receptors, in *The Receptors* (Conn P. M. eds), Vol.**4**: 171-212. Academic Press, New York.

Dearry, A., Gingrich, J. A., Falardeau, P., Fremeau, R. T., Bates, M. D., & Caron, M. G. (1990) Molecular cloning and expression of the gene for a human D1 dopamine receptor. *Nature* **347**, 72-76.

Dohlman, H. G., Thorner, J., Caron, M. G. & Lefkowitz, R. J. (1991) Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**, 653-688.

Dynan, W. S. (1986) Promoters for housekeeping genes. *Trends Genetics* **2**, 196-197.

Fremeau, R. T., Jr., Duncan, G. E., Fornaretto, M.-G., Dearry, A., Gingrich, J. A., Bresse, G. E. & Caron, M. G. (1991) Localization of D1 dopamine receptor mRNA in brain supports a role in cognitive, affective, and neuroendocrine

aspects of dopaminergic neurotransmission. *Proc. Natl. Acad. Sci. USA* **88**, 3772-3776.

Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z., Chase, T. N., Monsma, F. J., Jr.& Sibley, D. R. (1990) D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* **250**, 1429-1432.

Girault, J. A., Spampinato, U., Sauaki, H. E., Glowinski, J. & Besson, M. J. (1986) *In vivo* release of [^{3}H]r-aminobutyric acid in the neostriatum. *Neurosci.* **19**, 1101-1108.

Goodman, R. H. (1990) Regulation of neuropeptide gene expression. *Annu. Rev. Neurosci.* **13**, 111-127.

Grandy, D. K., Zhou, Q.-Y., Allen, L., Litt, R., Magenis, R. E., Civelli, O. & Litt, M. (1990) A Human D1 dopamine receptor gene is located on chromosome 5 at q35.1 and identifies an EcoRI RFLP. *Amer. J. Hum. Gen.* **47**, 828-834.

Graybiel, A. M., Moratalla, R. & Robertson, H. A. (1990) Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striasome-matrix compartments and limbic subdivisions of the striatum. *Proc. Natl. Acad. Sci. USA* **87**, 6912-6916.

Hershey, A. D., Dykema, P. E. & Krause, J. E. (1991) Organization, structure, and expression of the gene encoding the rat substance P receptor. *J. Biol. Chem.* **266**, 4366-4374.

Jensen, S. et al. (1992) Linkage analyses of the D1 dopamine receptor gene and manic depression, schizophrenia. Manuscript submitted.

Kebabian, J. W. & Calne, D. B. (1979) Multiple receptors for dopamine. *Nature 277*, 93-96.

Kozak, M. (1984) Compilation and analysis of sequences upstream from the translation start site in eukaryotic mRNAs. *Nucleic Acids Res.* **12**, 857-872.

Lankford, K. L., DeMello, F. G. & Klein, W. L. (1988) D1-type dopamine receptors inhibit growth cone mobility in cultured retina neurons: evidence that neurotransmitters act as morphogenic growth regulators in the developing central nervous system. *Proc. Natl. Acad. Sci. USA* **85**, 4567-4571.

Lee, W., Mitchell. P. & Tjian, R. (1987) Purified transcription factor Ap1 interacts with TPA-inducible enhancer elements. *Cell* **49**, 741-752.

Liu Y. F., Zhou, Q.-Y., Civelli, O. & Albert, P. (1992) Stimulation of the cloned human D1 receptor induces a cell-

specific, cholera toxin-sensitive calcium mobilization. Manuscript submitted.

Mahan, L. C., Burch, R. M., Monsma, F. J. Jr., & Sibley, D. (1990) Expression of striatal D1 dopamine receptors coupled to inositol phosphate production and Ca^{++} mobilization in Xenopus oocytes. *Proc. Natl. Acad. Sci. USA* **87**, 2196-2200.

Mansour, A., Meador-Woodruff, J. H., Zhou, Q.-Y., Civelli, O., Akil, H. & Watson, S. J. (1991) Comparison of D1 receptor binding and mRNA in the rat brain using receptor autoradiographic and in situ hybridization techniques. *Neurosci.* **45**, 359-371.

Mattingly, B. A., Rowleff, J. K., Graff, J. T. & Hatton, B. J. (1991) Effects of selective D1 and D2 dopamine antagonists on the development of behavioral sensitization to apomorphine. *Psychopharmacol.* **105**, 501-507.

Memo, M., Lovenberg, W. & Habauer, I. (1982) Agonist-induced subsensitivity of adenylate cyclase coupled with a dopamine receptor in slices from rat corpus striatum. *Proc. Natl. Acad. Sci. USA* **79**, 4456-4460.

Mitchell, P. J. & Tjian, R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371-378.

Moine, C. L., Normand, E. & Bloch, B. (1991) Phenotypical characterization of the rat striatal neurons expressing the D1 dopamine receptor gene. *Proc. Natl. Acad. Sci. USA* **88**, 4205-4209.

Monsma, F. J., Jr., Mahan, L. C., McVittie, L. D., Gerfen, C. R. & Sibley, D. R. (1990) Molecular cloning and expression of a D1 dopamine receptor linked to adenylyl cyclase activation. *Proc. Natl. Acad. Sci. USA* **87**, 6723-6727.

Montiminy, M. R., Gonazalez, G. A. & Yamamoto, K. K. (1990) Regulation of cAMP-inducible genes by CREB. *Trends Neurosci.* **13**, 184-188.

Mount, S. M. (1982) A catalogue of splice junction sequences. *Nucleic Acids Res.* **10**, 459-471.

Neumann, J. R., Morency, L. A. & Russian, K. O. (1987) A novel rapid assay for chloramphenicol acetyl transferase gene expression. *Biotechniques* **5**, 444-447.

O'Malley, K. L., Mack, K. J., Gandelman, K.-Y. & Todd, R. D. (1990) Organization and expression of the rat D2A receptor gene: Identification of alternative transcripts and a variant donor splice site. *Biochemistry* **29**, 1367-1371.

Piomelli, D., Pilon, C., Giros, B., Sokoloff, P., Martres, M.-P. & Schwartz, J.-C. (1991) Dopamine activation of the arachidonic acid cascade as a basis for D1/D2 receptor synergism. *Nature* **353**, 164-167.

Reubi, J.-C., Iverson, L. L. & Jessell, T. M. (1977) Dopamine selectively increase [3H]GABA release from slices of rat substantia nigra *in vitro*. *Nature* **268**, 652-654.

Rodrigues, P. D. S. & Dowling, J. E. (1990) Dopamine induces neurite retraction in retinal horizontal cells via diacylglycerol and protein kinase C. *Proc. Natl. Acad. Sci. USA* **87**, 9693-9697.

Sambrooks, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: a laboratory manual Cold Spring Harbor, New York.

Sawaguchi, T. & Goldman-Rakic, P. (1991) D1 dopamine receptors in frontal cortex: involvement in working memory. *Science* **251**, 947-950.

Sehgal, A., Patil, N. & Chao, M. (1988) A constitutive promoter directs expression of the nerve growth factor receptor gene. *Molec. Cell Biol.* **8**, 3160-3167.

Shaw, G. & Kamen, R. (1987) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46**, 659-667.

Sunahara, R. K., Niznik, H. B., Weiner, D. M., Stormann, T. M., Brann, M. R., Kennedy, J. L., Gelernter, J. E., Rozmahel, R., Yang, Y., Isreal, Y., Seeman, P. & O'Dowd, B. F. (1990) Human dopamine D1 receptor encoded by an intronless gene on chromosome 5. *Nature* **347**, 80-83.

Tsai, S. Y., Carstedt-Duke, J., Weigel, N. L., Dahlman, K., Gustafsson, J-A., Tsai, M.-J. & O'Malley, B. W. (1988) Molecular interaction of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. *Cell* **55**, 361-369.

Waddington, J. L. (1989) Functional interactions between D1 and D2 dopamine receptor system: their role in the regulation of psychomotor behavior, putative mechanisms and clinical relevance. *J. Psychopharmacol.* **3**, 54-63.

Waters, J. R., Bergstrom, D. A., Carlson, J. H., Chase, T. & Braun, A. (1987) D1 dopamine receptor activation required for postsynaptic expression of D2 agonist effects. *Science* **236**, 719-722.

Weiner, D. M., Levey, A. I., Sunahara, R. K., Niznik, H. B., O'Dowd, B. F., Seeman, P.& Brann, M. R. (1991) D1 and D2 dopamine receptor mRNA in the rat brain. *Proc. Natl. Acad. Sci. USA* **88**, 1859-1863.

Young, S. T., Porrino, L. J. & Zadarola, M. J. (1991) Cocaine induces striatal c-Fos-immunoreactive proteins via dopaminergic D1 receptors. *Proc. Natl. Acad. Sci. USA* **88**, 1291-1295.

Zhou, Q.-Y., Grandy, D. K., Thambi, L., Kushner, J. A., Van Tol, H.H.M., Cone, R., Pribnow, D., Salon, J., Bunzow, J. B., & Civelli, O. (1990) Cloning and expression of human and rat dopamine D1 receptors. *Nature* **347**, 76-80.

Molecular Cloning and Characterization of a Novel Adenosine Receptor: the A_3 Adenosine Receptor

Qun-Yong Zhou[#$], Chuanyu Li[#], Mark E. Olah[+], Robert A. Johnson[#], Gary L. Stiles[+], and Olivier Civelli[#@Ω].

[#]Vollum Institute for Advanced Biomedical Research
[$]Department of Biochemistry and
[@]Department of Cell Biology and Anatomy
Oregon Health Sciences University, Portland, OR 97201

[+]Departments of Medicine and Biochemistry
Duke University Medical Center
Durham, NC 27710

Running Title: Molecular Cloning of A_3 Adenosine Receptor

Ω To whom correspondence should be addressed

Abstract

We have previously reported the selective amplification of several rat striatal cDNA sequences that encode novel G-protein coupled receptors. One of these sequences (named R226) exhibited high sequence identities (58%) with the two previously cloned adenosine receptors RDC7 (A1) and RDC8 (A2). A full-length cDNA clone for R226 has been isolated from a rat brain cDNA library. The cDNA clone encodes a protein of 320 amino acids that can be organized into seven transmembrane stretches. R226 has been expressed in COS-7 and CHO cells and membranes from the transfected cells were screened with adenosine receptor radioligands. R226 could bind the nonselective adenosine agonist [^{3}H]NECA and A1-selective agonist [^{125}I]APNEA but not A1-selective antagonists [^{3}H]DPCPX and [^{3}H]XAC or the A2-selective agonist ligands [^{3}H]CGS21680 and [^{125}I]PAPA-APEC. Extensive characterization with [^{125}I]APNEA showed that R226 binds [^{125}I]APNEA with high affinity (Kd=17 nM) and the specific [^{125}I]APNEA binding could be inhibited by adenosine ligands with a potency order of R-PIA = NECA > S-PIA > adenosine > ATP = ADP but not by antagonists XAC, IBMX and DPCPX. In R226 stably transfected CHO cells, adenosine agonists R-PIA, NECA, and CGS21680 inhibited by 40-50% the forskolin-stimulated cAMP accumulation through a pertussis toxin-sensitive G protein with EC50 of 18±5.6 nM, 23±3.5 nM, and 144±34 nM, respectively. Based on these observations we conclude that R226 encodes an adenosine receptor with non-A1 and non-A2 specificity and thus name it the A3 adenosine receptor. mRNA analyses revealed that the highest expression of R226 was in the testis and low level mRNAs were also found in the lung, kidneys, heart, and some parts of the central nervous system such as cortex, striatum and olfactory bulb. The high expression level of the A3 receptor in the testis suggests a possible role for adenosine in reproduction.

Introduction

Adenosine modulates diverse physiological functions including induction of sedation, vasodilatation, suppression of cardic rate and contractility, inhibition of platelet aggregability, stimulation of gluconeogenesis and inhibition of lipolysis (1-3). Based on biochemical and pharmacological criterion, two subtypes of adenosine receptors A1 and A2 have been differentiated, which inhibit and stimulate adenylate cyclase respectively (1,3). Substantial progress has been made concerning the biochemical and pharmacological properties of adenosine receptors including ligand binding characteristics, glycoprotein nature, and molecular regulation (1-3). Besides its effects on adenylate cyclase, adenosine has been shown to open K^+ channels, reduce flux through Ca^{++} channels, and inhibit or stimulate phosphoinositide turnover through receptor-mediated mechanisms (4-7). In addition, the A1 adenosine receptor has been purified to homogeneity from rat and bovine brains (8,9).

Recently, the cDNAs that encode A1 and A2 adenosine receptors have been cloned by a PCR-based strategy (10-14). Molecular cloning of A1 and A2 receptors revealed that they both belong to the superfamily of G-protein coupled receptors. Physiological and pharmacological studies, however, have suggested a subtype heterogeneity of adenosine receptor besides the A1 and A2 classification (15-18). A new variant of the A1AR has recently been cloned from a bovine brain cDNA library that has distinct pharmacologic properties from the rat and dog A1AR but has 90% homology at the protein level (19). We report here the cloning, expression and functional characterization of a novel adenosine receptor. We present its nucleotide and deduced amino acid sequence, its tissue distribution, its pharmacological characterization and its ability to inhibit forskolin-stimulated cAMP accumulation and we conclude that R226 is distinct enough from the A1 and A2

receptors to be called the A3 adenosine receptor. The A3 receptor appears closer in structure and function to the A1AR than the A2AR.

Materials and Methods

PCR amplification, library screening and sequencing. Rat striatal cDNA mixture was subjected to 30 cycles of PCR amplification with a pair of degenerate oligonucleotide primers (21). The PCR products sizing from 400 to 750 bp were subcloned into M13 and sequenced (22). One fragment, named PCR226, with sequence homologous to RDC7 and RDC8 (10) was identified. Random-primed ^{32}P-labelled PCR226 was used to screen a rat brain cDNA library in λgt11 and a rat genomic library in λDASH by plaque hybridization (20). One cDNA clone was identified and its cDNA insert was subcloned into pGemblue (resulting in pGem226) and sequenced. Two identical positive genomic phages were also plaque purified and were characterized by Southern blotting hybridization and partially sequenced.

DNA transfection, membrane preparation and receptor binding assays. The full coding region of R226 cDNA insert was cut out from pGem226 and subcloned into HindIII/XbaI sites of Rc-RSV expression vector (Rc-RSV226) and HindIII/SmaI sites of pBC12BI (pBC226). For transient expression, 45 μg pBC226 DNA were transfected into each 150mm dish of COS-7 cells according to Chen and Okayama method (23) and cells were harvested 48 hrs later. For stable expression, 1 μg mini-prepared RcRSV226 plasmid DNA was further purified by Prep-A-Gene kit of Bio-Rad and transfected into CHO cells by the CaPO4 method (20). CHO cells were selected with neomycin (G418, 700 μg/ml) and screened for expression of R226 by Northern blot hybridization (20). Membrane preparation and radioligand binding assays were performed as previously described except that adenosine deaminase was not

included when adenosine competition curves were performed (21,24).

cAMP measurement. Cells were grown to about 80% confluence in 150 mm dishes and washed twice with Ca^{++} and Mg^{++}-free PBS buffer and detached from plates with PBS buffer containing 0.02% EDTA. Cells were spun down at 800 rpm for 10 min at 4°C and resuspended in appropriate volume of KRH buffer (140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 6 mM glucose, 25 mM Hepes-NaOH, pH 7.4). The cells were washed once with KRH buffer and resuspended in KRH buffer at a concentration of 10^7/ml. 100 µl cell suspension was added to each glass tube containing 100 µl KRH + 200 µM Ro 20-1724 and incubated at 37°C for 10 min. Prewarmed 200 µl KRH containing 200 µM Ro 20-1724 and test drugs were then added to cells and mixed. After incubation at 37°C for 20 min, 400 µl 0.5 mM NaAc, pH 6.2 were added and the glass tubes were transferred to a boiling water bath. Boiling was for 20 min, and then the tubes were cooled to room temperature and centrifuged at 3000 rpm for 15 min. 50 µl of supernatants were assayed for cAMP levels (25). For pertussis toxin pretreatment, aseptic pertussis toxin (Sigma) was added to the medium to a final concentration of 100 ng/ml and maintained for approximately 18 hours.

Reverse transcription-PCR. 2 µg total RNAs isolated from different rat tissues were reverse transcribed (20). The single-strand cDNA products were denatured and subjected to limited PCR amplification (27 cycles) with two primers (TTCCAGCTGAAGCTTCTC as 5' primer and GGTGGAGCTGTTTTGAGA as 3' primer). Each PCR cycle consisted of denaturing at 95°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 90 seconds. PCR products were then run on the 1.2% agarose gel and examined by ethidium bromide staining. A standard curve was also developed to test the linearity of the PCR amplification. pGem226 (above) was linearized by

XbaI, and RNA was synthesized with T7 RNA polymerase. 640,320,160,80,40,20,and 10 fg of the *in vitro* synthesized template RNA were reverse transcribed and PCR amplified as described above. Ethidium bromide staining of the PCR products indicated that the amount of amplified products was approximately proportional to the amount of template added.

Results

As part of our efforts to clone new G-protein coupled receptors, rat striatal cDNA was used as template in a PCR amplification using a pair of degenerate oligonucleotides corresponding to the putative third and sixth transmembrane regions of G-protein coupled receptors (21). The PCR products were characterized by sequencing. Five novel sequences that represent putative new members of the G-protein coupled receptor were identified (data not shown). One of these fragments, R226, exhibited pronounced sequence homology with the two previously cloned adenosine receptors RDC7 and RDC8 (10-14).

The R226 fragment was subsequently used to screen a rat brain cDNA library. Out of 10^6 cDNA clones screened, one positive clone with an insert of 1.9 kb was isolated and its complete nucleotide sequence was determined (Fig.1). The longest open reading frame of this cDNA codes a protein of 320 amino acids with a calculated molecular weight of 36,644 kD. This protein is among the smallest members of the G-protein coupled receptor family (26) as is the A1 receptor (RDC7). A Kozak consensus sequence (27) is found around the presumed translation start codon (Fig.1B).

Hydrophobicity analysis of the deduced amino acid sequence showed that the protein contains seven hydrophobic stretches of 21 to 26 amino acids. A computer sequence search revealed that R226 is significantly homologous to the cloned adenosine receptors (Fig.1C) In putative transmembrane domains, R226

has 58% amino acids identical to that of A1 receptor (RDC7) and 57% identical to that of A2 receptor (RDC8). Of these three receptors, about 46% of the amino acid residues are identical in the putative transmembrane domains. These levels of sequence identities are similar to what has been found for adrenergic and dopaminergic receptor subfamilies (26,28). The high sequence similarity among A1, A2 and R226 suggested that they might also belong to the same subclass of G-protein coupled receptor. Furthermore, they all possess a putative N-glycosylation site in the second extracellular loop (Fig.1B). However, in contrast to A1 and A2 adenosine receptors, which lack N-glycosylation sites in the N-terminal, two potential N-glycosylation sites (Asn-4 and Asn-5) were found in the N-terminal of R226 (Fig.1B). A search for potential phosphorylation sites revealed the presence of three potential phosphorylation sites (consensus sequence S/T-X-R/K) by protein kinase C (29) but no consensus sequence (R/K-R/K-X-S/T) for a protein kinase A phosphorylation site (30). Interestingly, one potential phosphorylation site by casein kinase II (S/T-X-X-E/D) was found in the short C-terminus of R226 (31). These potential phosphorylation sites could be involved in receptor function regulation (30).

Partial characterization of rat genomic clones of R226 revealed the existence of at least one intron (>2 kb) with a donor/acceptor site sequence of TTTTCCTCCCCCCATTCAAACCAG/AT. This splice site is located just outside the third transmembrane domain of R226 and all the mRNA sequence of R226 after this splice site is contained in a single exon (Fig.1B). Evolutionally, this splice site seems conserved in several members of the G-protein coupled receptor family that contain introns (32).

Initial binding assays of transiently transfected COS-7 cell membrane preparations with the nonselective adenosine radioligand [^{3}H]NECA showed a saturable (Bmax=550 fmol/mg

protein) and high affinity (Kd=50 nM) binding and the [^{3}H]NECA binding could be inhibited to different degrees by adenosine ligands (Data not shown). This result suggested that R226 might encode an adenosine receptor. To further characterize this receptor and also to avoid the low level of endogenous A2 receptor in COS-7 cells, we stably expressed R226 in CHO cells, which do not express endogenous adenosine receptors as judged by [^{3}H]NECA binding (Data not shown). Using membranes from stably transfected CHO cells, we assessed the ability of a variety of radioligands including the A1 selective agonist [^{125}I]APNEA, the A1 selective antagonists [^{3}H]DPCPX and [^{3}H]XAC, the A2 selective agonists [^{3}H]CGS21680 and [^{125}I]PAPA-APEC to specifically bind to the R226 receptor. Only [^{125}I]APNEA gave specific binding as defined by R-PIA (10 μM) or NECA (10 μM). Using direct saturation curves or dilution saturation curves, [^{125}I]APNEA bound with high affinity (15.5 ± 2.4 nM) and in a saturable manner (225 fmol/mg). This affinity is about ten fold lower than that found for typical A1 receptors (33). A representative Scatchard plot is shown in Fig.2. R226 was further characterized in competition assays employing a large series of receptor ligands and [^{125}I]APNEA as the labelled ligand. Shown in Fig.3A is the competition of the prototypic adenosine analogs used to define A1 and A2 receptor subtypes. R-PIA and NECA were equally potent in competing for binding with IC50 of 63 ± 19 nM and 74 ± 23 nM, respectively, while S-PIA had an IC50 of 1140 ± 490 nM. The presumed endogenous hormone adenosine competes with an IC50 of 30 ± 4 μM. A constant feature of all previously known adenosine receptors is that alkylxanthines are receptor antagonists. In contrast, none of the alkylxanthines tested including IBMX, DPCPX and XAC compete even at 100 μM concentrations. Since the above pharmacology and ligand affinities are not consistent with the expected A1 or A2 receptor pharmacology, we next tested whether R226 might be a purinergic P2 receptors or other nucleoside or nucleotide binding protein (34). ATP at 100 μM

inhibits only 50% of specific [^{125}I]APNEA binding while ADP at the same concentration inhibits only 25% of specific binding (Fig.3B). App(NH)p, AMP-PCP, 2-CH_3-S-ATP, UTP, cAMP and 2-deoxyadenosine all failed to inhibit binding by more than 20% at 1 mM. The following ligands all failed to compete with [^{125}I]APNEA binding at 100 µM: isoproterenol, carbachol, phentolamine, serotonin and dopamine. However, Gpp(NH)p, a nonhydrolyzable analog of GTP, effectively competed for 60-70% of [^{125}I]APNEA specific binding with an IC50 approximately 1 µM (Fig.3B). This is very reminiscent of what is observed for the effects of guanine nucleotides on A1 receptor-agonist binding (33) but totally distinct from that observed in the A2 receptor system (35).

To analyze the biological activity of the cloned adenosine receptor, we studied its ability to couple to second messenger systems. R226 stably transfected CHO cells were used to study the effects of adenosine agonists on cAMP accumulation. Incubation of wild type and R226 stably transfected CHO cells with 1 µM forskolin resulted in a 15-fold increase in cellular cAMP levels. In wild type CHO cells, adenosine agonists had no effect on forskolin-stimulated cAMP production (data not shown). Addition of adenosine receptor agonists R-PIA (100 nM), NECA (100 nM), CGS21680 (100 nM), and adenosine (100 µM) on R226 stably transfected CHO cells produced 20-50% inhibition of forskolin-stimulated cAMP accumulation (Fig.4A). ATP and ADP had no effects at 100 µM concentration. Further experiments showed that inhibition of forskolin-stimulated cAMP production by adenosine agonists was dose-dependent with EC50 of 18 ± 5.6 nM for R-PIA, 23 ± 3.5 nM for NECA, 144 ± 34 nM for CGS21680 and 6.5 ± 2.1 µM for adenosine (Fig.5). The maximal inhibition for R-PIA, NECA and CGS21680 was 40% to 50%, although adenosine itself had not reached its maximal effect (Fig.5). The inhibitory effect of NECA on forskolin-stimulated cAMP production could not be reversed by

incubation with 10 μM DPCPX, a very selective A1 adenosine receptor antagonist (Fig.4B). This result agrees well with the binding studies where R226 did not bind any alkylxanthine type antagonist. These data indicate that the cloned adenosine receptor is negatively coupled to adenylate cyclase.

We then analyzed whether the inhibition of adenylate cyclase induced by the stimulation of the cloned adenosine receptor is transduced through a pertussis toxin-sensitive G protein. Fig.4B shows that pretreatment with pertussis toxin almost completely abolished the inhibitory effect of R-PIA and NECA on forskolin-stimulated cAMP production. This result indicated that this novel adenosine receptor inhibits adenylate cyclase through interaction with a pertusiss toxin-sensitive G protein. This conclusion was also supported by the observation that [^{125}I]APNEA binding could be inhibited by Gpp(NH)p (Fig.3B).

To gain further insight into the possible responses associated with this novel adenosine receptor, we have examined the tissue distribution of its corresponding messenger RNA by RT-PCR (20). Two primer sequences located in the N-terminal and the second extracellular loop were chosen. These primers were chosen so that: (1) they cross the intronic sequence, which is at least 2kb in length, to avoid detection of possible genomic DNA contamination; (2) they can discriminate R226 from A2 and A1 adenosine receptors. As shown in Fig.6, highest expression was observed in the testis. The kidneys, the lungs and the heart exhibited moderate levels of expression. In the central nervous system, low level expression was detected in cortex, striatum, and olfactory bulb. It is interesting to compare the tissue distribution of R226 with that of A1 and A2 receptors. A1 and A2 adenosine receptors are highly expressed in the brain regions such as cerebral cortex, hippocampus, cerebellum, and

thalamus (10-14). In contrast, R226 has relatively low expression in the central nervous system and is mainly expressed in peripheral tissues. The high expression level of R226 found in the testis suggests that adenosine might play a role in reproduction.

Discussion

On the basis of its sequence, the R226 protein belongs to the superfamily of the G protein-coupled receptors and to the family of the adenosine receptors but is clearly distinct from the A1 and A2 adenosine receptors. The sequence of R226 is the same as the recently reported tgpcr1 cDNA (36). However, the tgpcr1 mRNA was found only in the testis and its pharmacological profile and biological activity of the corresponding protein was not studied (36). The sequence identity comparison suggested that R226 is probably not a subtype of either A1 or A2 adenosine receptors. Within the putative transmembrane domains, A1 (RDC7) and A2 (RDC8) share 63% amino acid identity. If R226 belongs to a subtype of either A1 or A2, we expect the amino acid sequence identities between R226 and A1 or A2 will be greater than 63% (26,28). However, the amino acid sequence identity in transmembrane spanning regions between R226 and A1 is 58% and between R226 and A2 is 57%.

We found that the R226 receptor binds adenosine receptor agonists with reasonable affinity but without a pharmacological profile consistent with either an A1 or A2 adenosine receptor (Fig.2,3). In addition, R226 did not bind traditional adenosine receptor antagonists making it totally distinct from the A1 and A2 adenosine receptors (2,6). The R226 receptor is able to inhibit adenylyl cyclase activity through a pertussis toxin-sensitive G protein (Fig.4,5). In addition, the binding of [^{125}I]APNEA to R226 is modulated by guanine nucleotides (Fig.3B). The reduction in agonist

binding by 60-70% in membranes is reminiscent of that described for the A1 adenosine receptor (33) but not the A2 adenosine receptor (35). Its ability to inhibit adenylyl cyclase also reflects its similarity to A1 receptor activity, yet it is not blocked by A1-specific antagonists (Fig.4B). This suggests that although R226 is closer to the A1 adenosine receptor in function than the A2 adenosine receptor it is not close enough in pharmacological properties or tissue distribution to warrant making it a subtype of A1 adenosine receptor and, therefore, should be considered distinct from A1 and A2 adenosine receptors. The affinities of adenine nucleotides and nucleosides and UTP for the receptor also suggests that this receptor is not a known subtype of P_2 receptors (34). We conclude that R226 is a novel adenosine receptor and we propose to classify it as the A3 adenosine receptor. We must immediately point out that the relationship between this receptor and the putative A3 adenosine receptor postulated by Ribeirio et al. (16) is totally unknown. Ribeiro and Sebastiao have suggested that there is a distinct adenosine receptor in the brain that is coupled to Ca^{++} metabolism but no definitive characterization was provided. In addition, this putative receptor is inhibited by alkylxanthines making it distinct from the A3 adenosine receptor reported here. We, therefore, propose that as new adenosine receptors are discovered and characterized which clearly are not subtypes of known adenosine receptors, they are simply assigned increasing numerical values and hence this receptor is the A3 adenosine receptor.

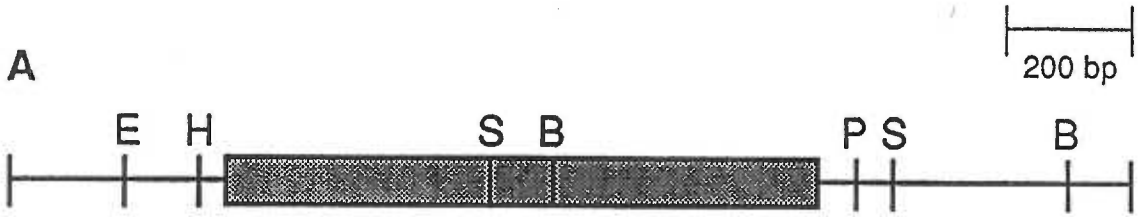


Fig. 1A

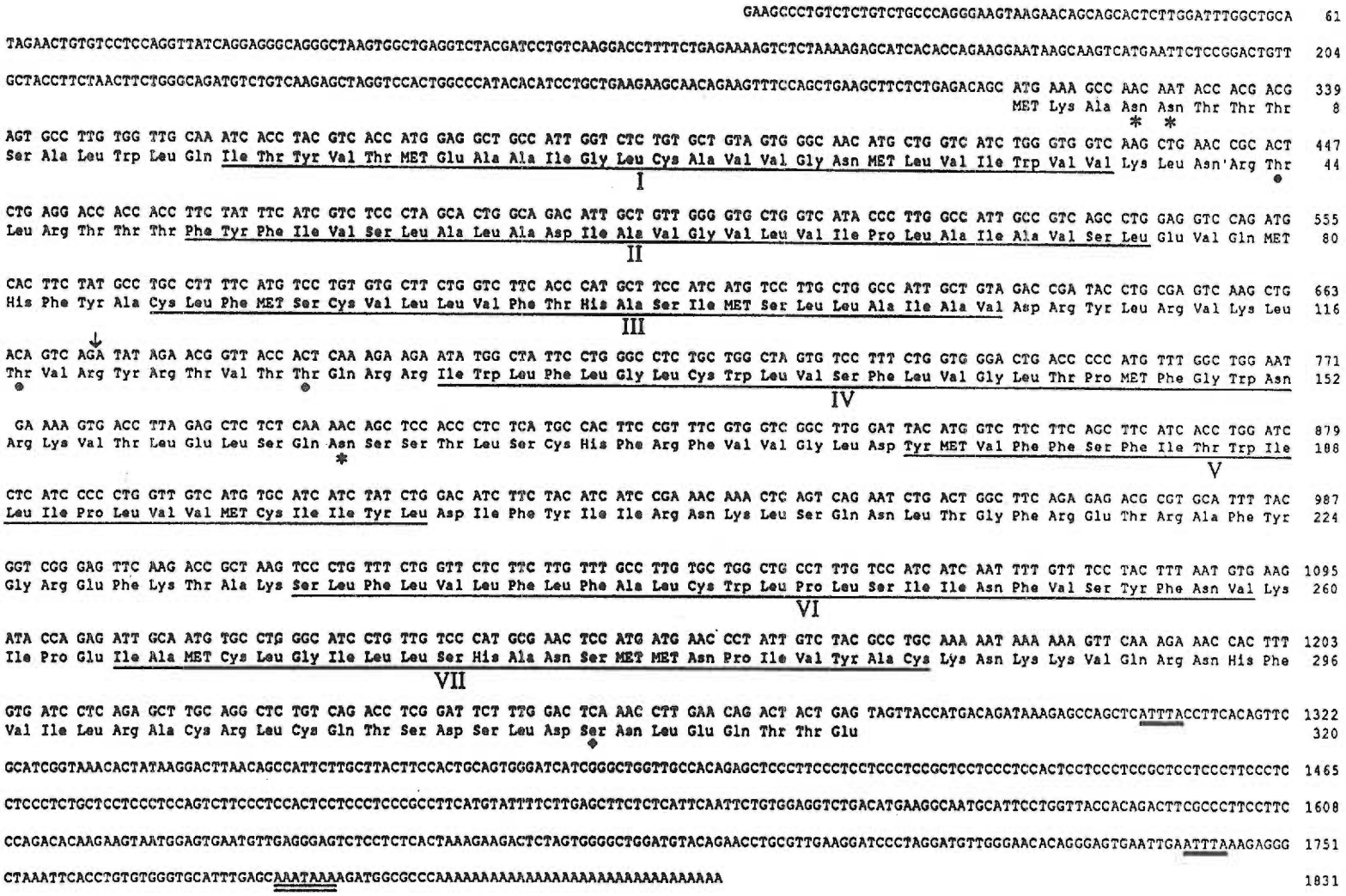

GAAGCCCTGTCTCTGTCTGCCCAGGGAAGTAAGAACAGCAGCACTCTTGGATTTGGCTGCA 61
TAGAACTGTGTCCTCCAGGTTATCAGGAGGGCAGGGCTAAGTGGCTGAGGTCTACGATCCTGTCAAGGACCTTTTCTGAGAAAAGTCTCTAAAAGAGCATCACACCAGAAGGAATAAGCAAGTCATGAATTCTCCGGACTGTT 204
GCTACCTTCTAACTTCTGGGCAGATGTCTGTCAAGAGCTAGGTCCACTGGCCCATACACATCCTGCTGAAGAAGCAACAGAAGTTTCCAGCTGAAGCTTCTCTGAGACAGC ATG AAA GCC AAC AAT ACC ACG ACG 339
MET Lys Ala Asn Asn Thr Thr Thr 8
AGT GCC TTG TGG TTG CAA ATC ACC TAC GTC ACC ATG GAG GCT GCC ATT GGT CTC TGT GCT GTA GTG GGC AAC ATG CTG GTC ATC TGG GTG GTC AAG CTG AAC CGC ACT 447
Ser Ala Leu Trp Leu Gln Ile Thr Tyr Val Thr MET Glu Ala Ala Ile Gly Leu Cys Ala Val Val Gly Asn MET Leu Val Ile Trp Val Val Lys Leu Asn Arg Thr 44
I
CTG AGG ACC ACC ACC TTC TAT TTC ATC GTC TCC CTA GCA CTG GCA GAC ATT GCT GTT GGG GTG CTG GTC ATA CCC TTG GCC ATT GCC GTC AGC CTG GAG GTC CAG ATG 555
Leu Arg Thr Thr Thr Phe Tyr Phe Ile Val Ser Leu Ala Leu Ala Asp Ile Ala Val Gly Val Leu Val Ile Pro Leu Ala Ile Ala Val Ser Leu Glu Val Gln MET 80
II
CAC TTC TAT GCC TGC CTT TTC ATG TCC TGT GTG CTT CTG GTC TTC ACC CAT GCT TCC ATC ATG TCC TTG CTG GCC ATT GCT GTA GAC CGA TAC CTG CGA GTC AAG CTG 663
His Phe Tyr Ala Cys Leu Phe MET Ser Cys Val Leu Leu Val Phe Thr His Ala Ser Ile MET Ser Leu Leu Ala Ile Ala Val Asp Arg Tyr Leu Arg Val Lys Leu 116
III
ACA GTC AGA TAT AGA ACG GTT ACC ACT CAA AGA AGA ATA TGG CTA TTC CTG GGC CTC TGC TGG CTA GTG TCC TTT CTG GTG GGA CTG ACC CCC ATG TTT GGC TGG AAT 771
Thr Val Arg Tyr Arg Thr Val Thr Thr Gln Arg Arg Ile Trp Leu Phe Leu Gly Leu Cys Trp Leu Val Ser Phe Leu Val Gly Leu Thr Pro MET Phe Gly Trp Asn 152
IV
GA AAA GTG ACC TTA GAG CTC TCT CAA AAC AGC TCC ACC CTC TCA TGC CAC TTC CGT TTC GTG GTC GGC TTG GAT TAC ATG GTC TTC TTC AGC TTC ATC ACC TGG ATC 879
Arg Lys Val Thr Leu Glu Leu Ser Gln Asn Ser Ser Thr Leu Ser Cys His Phe Arg Phe Val Val Gly Leu Asp Tyr MET Val Phe Phe Ser Phe Ile Thr Trp Ile 188
V
CTC ATC CCC CTG GTT GTC ATG TGC ATC ATC TAT CTG GAC ATC TTC TAC ATC ATC CGA AAC AAA CTC AGT CAG AAT CTG ACT GGC TTC AGA GAG ACG CGT GCA TTT TAC 987
Leu Ile Pro Leu Val Val MET Cys Ile Ile Tyr Leu Asp Ile Phe Tyr Ile Ile Arg Asn Lys Leu Ser Gln Asn Leu Thr Gly Phe Arg Glu Thr Arg Ala Phe Tyr 224
GGT CGG GAG TTC AAG ACC GCT AAG TCC CTG TTT CTG GTT CTC TTC TTG TTT GCC TTG TGC TGG CTG CCT TTG TCC ATC ATC AAT TTT GTT TCC TAC TTT AAT GTG AAG 1095
Gly Arg Glu Phe Lys Thr Ala Lys Ser Leu Phe Leu Val Leu Phe Leu Phe Ala Leu Cys Trp Leu Pro Leu Ser Ile Ile Asn Phe Val Ser Tyr Phe Asn Val Lys 260
VI
ATA CCA GAG ATT GCA ATG TGC CTG GGC ATC CTG TTG TCC CAT GCG AAC TCC ATG ATG AAC CCT ATT GTC TAC GCC TGC AAA AAT AAA AAA GTT CAA AGA AAC CAC TTT 1203
Ile Pro Glu Ile Ala MET Cys Leu Gly Ile Leu Leu Ser His Ala Asn Ser MET MET Asn Pro Ile Val Tyr Ala Cys Lys Asn Lys Lys Val Gln Arg Asn His Phe 296
VII
GTG ATC CTC AGA GCT TGC AGG CTC TGT CAG ACC TCG GAT TCT TTG GAC TCA AAC CTT GAA CAG ACT ACT GAG TAGTTACCATGACAGATAAAGAGCCAGCTCATTTACCTTCACAGTTC 1322
Val Ile Leu Arg Ala Cys Arg Leu Cys Gln Thr Ser Asp Ser Leu Asp Ser Asn Leu Glu Gln Thr Thr Glu 320
GCATCGGTAAACACTATAAGGACTTAACAGCCATTCTTGCTTACTTCCACTGCAGTGGGATCATCGGGGCTGGTTGCCACAGAGCTCCCTTCCCTCCTCCCTCCGCTCCTCCCTCCACTCCTCCCTCCGCTCCTCCCTTCCCTC 1465
CTCCCTCTGCTCCTCCCTCCAGTCTTCCCTCCACTCCTCCCTCCCGCCTTCATGTATTTTCTTGAGCTTCTCTCATTCAATTCTGTGGAGGTCTGACATGAAGGCAATGCATTCCTGGTTACCACAGACTTCGCCCTTCCTTC 1608
CCAGACACAAGAAGTAATGGAGTGAATGTTGAGGGAGTCTCCTCTCACTAAAGAAGACTCTAGTGGGGCTGGATGTACAGAACCTGCGTTGAAGGATCCCTAGGATGTTGGGAACACAGGGAGTGAATTGAATTTAAAGAGGG 1751
CTAAATTCACCTGTGTGGGTGCATTTGAGCAAATAAAAGATGGCGCCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1831

Fig. 1B

AMINO ACID SEQUENCE ALIGNMENT

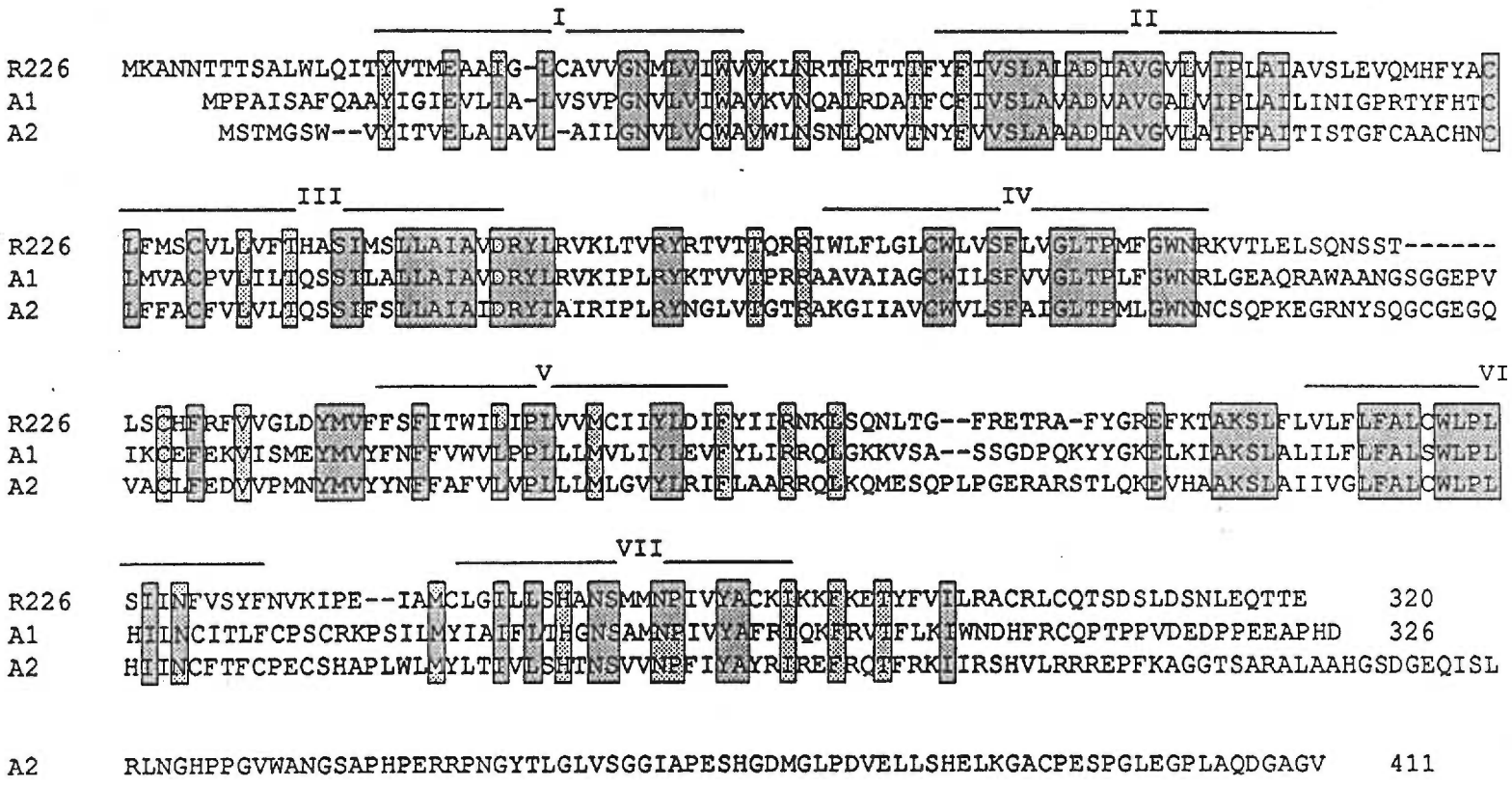


Fig. 1C

Figures and legends

Fig.1 **A**. Restriction map of R226 receptor cDNA. E: EcoRI, H: HindIII, B: BamHI, P: PstI, S: SacI. The open reading frame (ORF) is indicated by the filled box. **B**. Nucleotide and deduced amino acid sequence of R226 cDNA. Numbering starts with the first nucleotide of the cDNA. Putative transmembrane domains are underlined. A putative polyadenylylation site has two lines below it. The potential N-glycosylation sites are indicated by stars (*). The arrow indicates a splice site. The RNA destability consensus sequence AUUUA signals have bold lines below them. The potential protein kinase C phoshorylation sites and casein kinase II phosphorylation site are indicated by filled dots and diamond respectively. **C**. Amino acid sequence alignment of R226, RDC7 and RDC8. The putative transmembrane domains are boxed and bracketed and labelled by Roman numerals. The shaded amino acid residues indicate they are conserved in all three adenosine receptors.

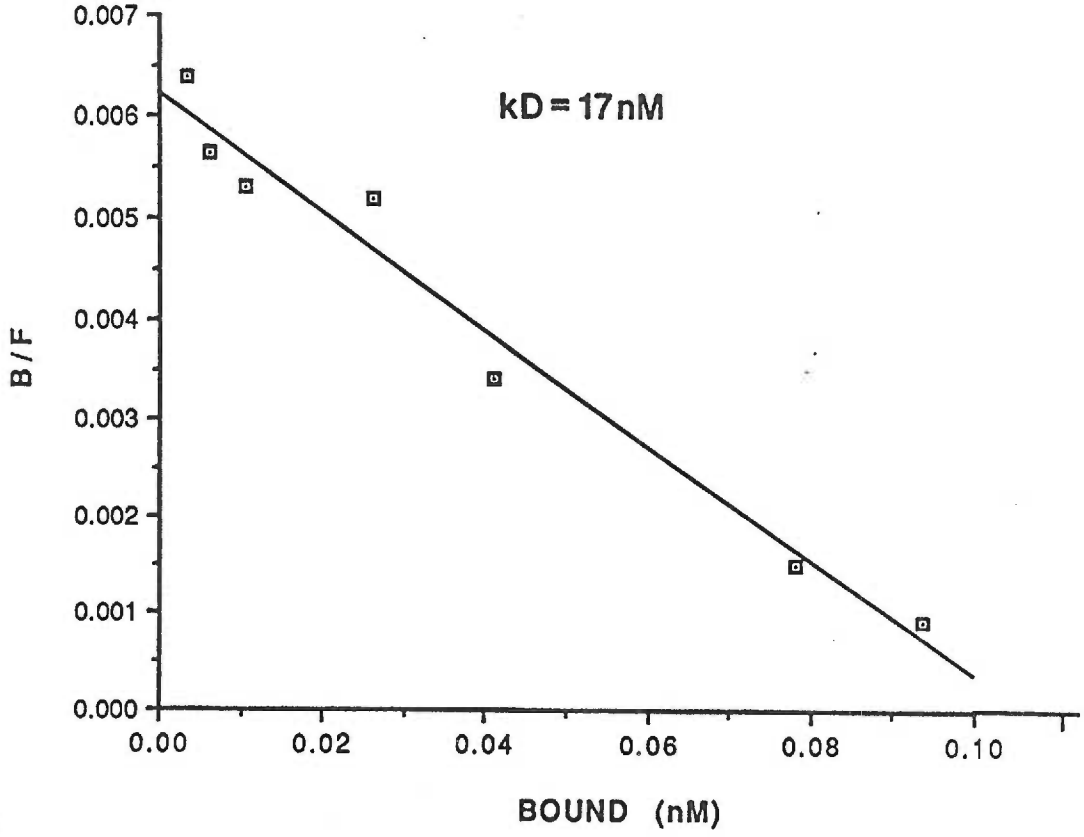

kD = 17nM
B/F
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
0.00
0.02
0.04
0.06
0.08
0.10
BOUND (nM)

Fig. 2

Fig.2 Scatchard analysis of [^{125}I]APNEA binding to membranes prepared from stably transfected CHO cells. Membranes were prepared as described in Methods. [^{125}I]APNEA was directly added up to 2 nM and then diluted with I-APNEA up to 100 nM. Nonspecific binding was defined by 10 μM R-PIA. The experiment was repeated five times.

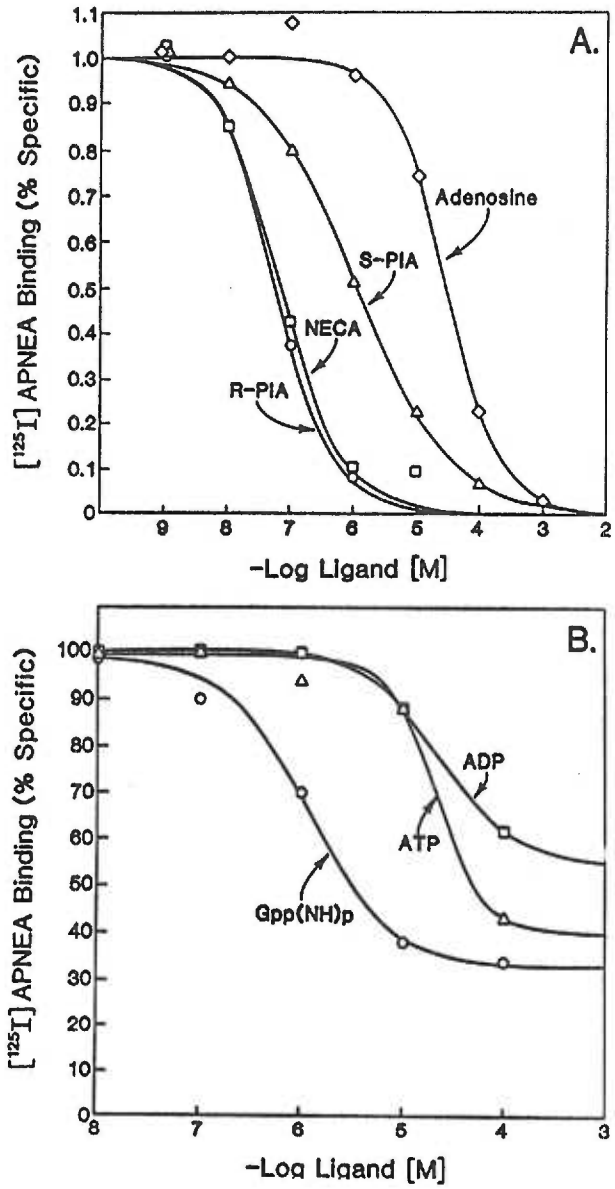

A.
[125I] APNEA Binding (% Specific)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
9
8
7
6
5
4
3
2
-Log Ligand [M]
Adenosine
S-PIA
NECA
R-PIA
B.
[125I] APNEA Binding (% Specific)
100
90
80
70
60
50
40
30
20
10
0
8
7
6
5
4
3
-Log Ligand [M]
ADP
ATP
Gpp(NH)p

Fig. 3

Fig.3 **A**. Competition curves of adenosine receptor agonists versus [125I]APNEA. Membranes from stably transfected CHO cells were prepared as described in Methods. [^{125}I]APNEA was present at 0.5 nM and competitors were present at the indicated concentration. **B**. Competition for [^{125}I]APNEA by Gpp(NH)p and the P2 receptor agonists ATP and ADP. Competition curves were replicated three to five times depending upon the ligands used. The Hill coefficients were near unity.

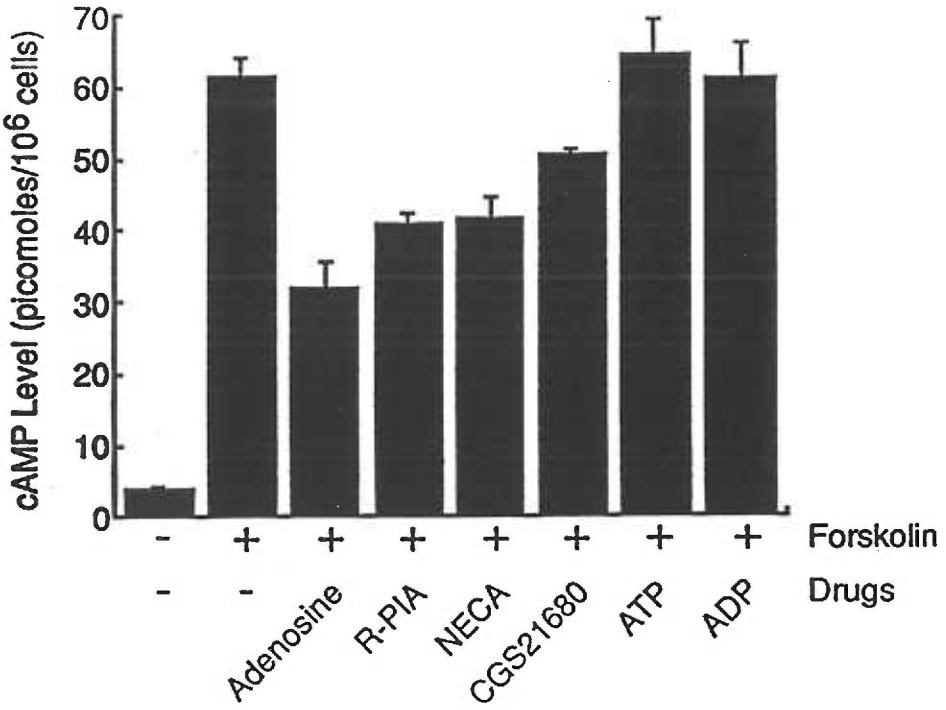

cAMP Level (picomoles/10^6 cells)
70
60
50
40
30
20
10
0
- + + + + + + +
Forskolin
- - Adenosine R-PIA NECA CGS21680 ATP ADP
Drugs

Fig. 4A

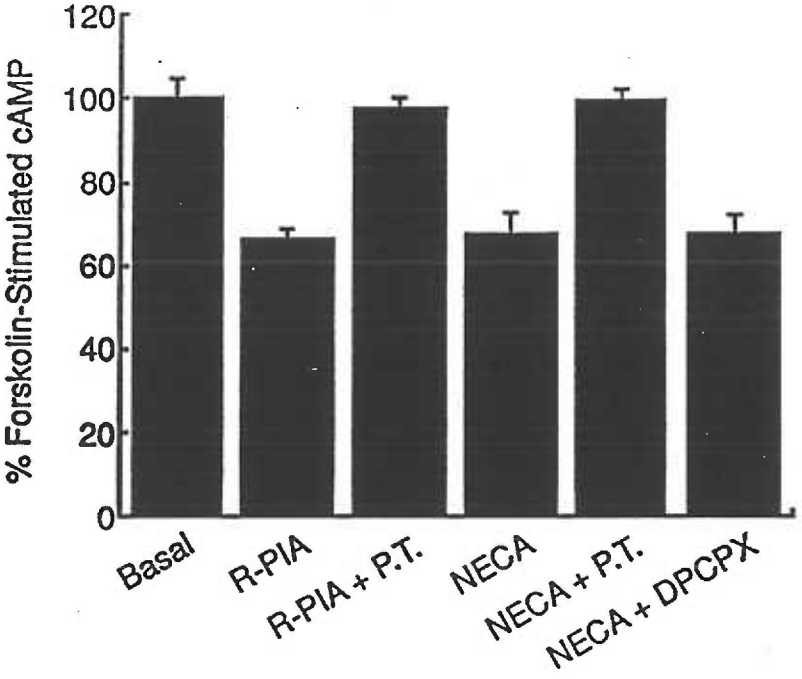

% Forskolin-Stimulated cAMP
120
100
80
60
40
20
0
Basal
R-PIA
R-PIA + P.T.
NECA
NECA + P.T.
NECA + DPCPX

Fig. 4B

Fig.4 **A**. Effects of adenosine agonists on forskolin-stimulated cAMP accumulation in CHO cells stably transfected by R226. The concentration of adenosine agonists used are 100 nM (R-PIA, NECA and CGS21680) and 100 μM (adenosine, ATP and ADP) and the concentration of forskolin is 1 μM. Values are obtained from triplicate plates and shown as mean±s.e. **B**. Effects of pertussis toxin pretreatment on forskolin-stimulated cAMP accumulation in R226 stably transfected CHO cells. The cells are either pretreated or not with 100 ng/ml pertussis toxin for 18 hours. The concentrations of forskolin and adenosine agonists used are the same as **A**.

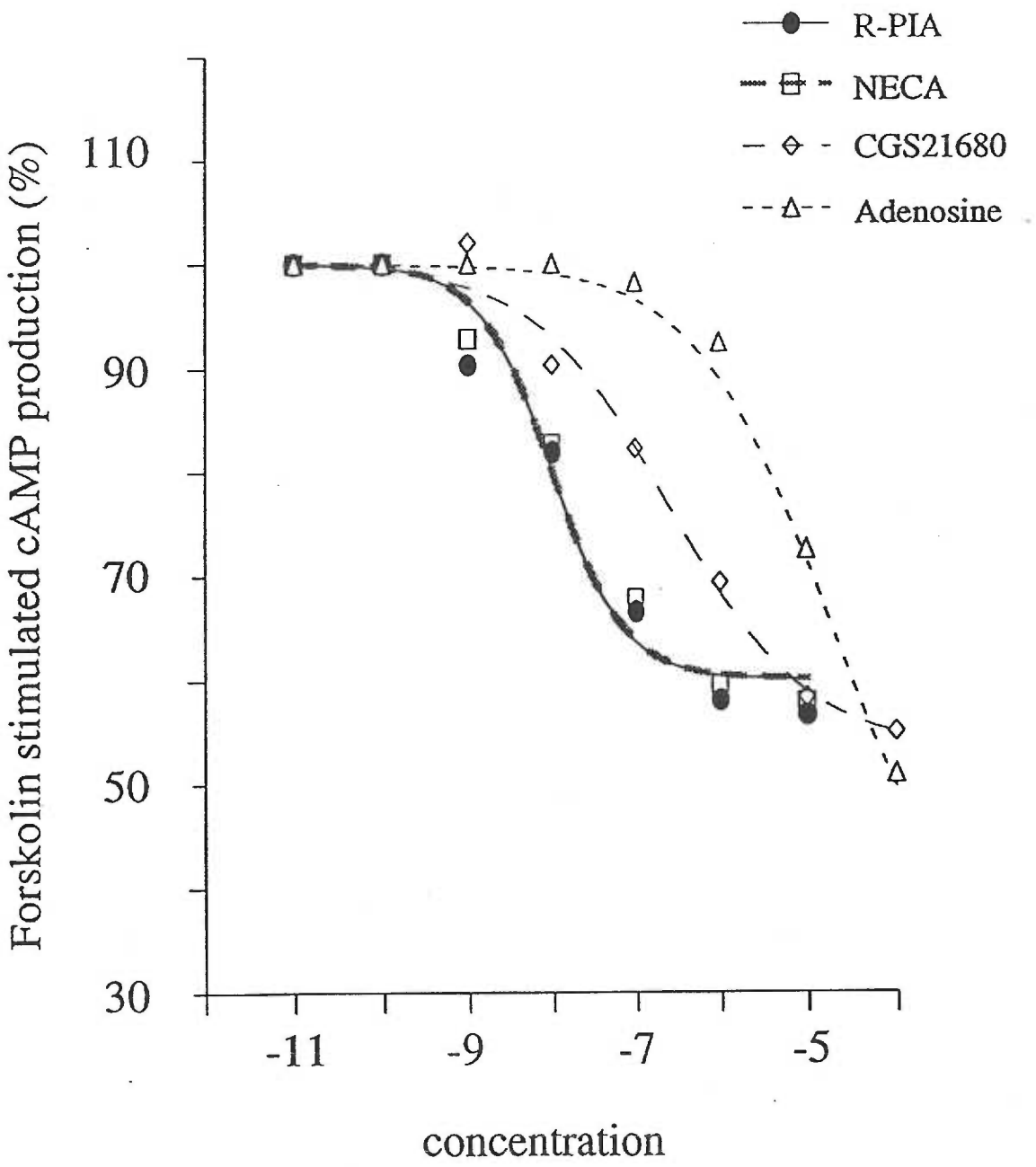

R-PIA
NECA
CGS21680
Adenosine
Forskolin stimulated cAMP production (%)
110
90
70
50
30
-11
-9
-7
-5
concentration

Fig.5

Fig.5 Dose-dependent inhibition by adenosine agonists of forskolin-stimulated cAMP accumulation in CHO cells stably transfected with R226. Results are shown as percentage of maximal stimulation of cAMP accumulation observed with 1 μM forskolin alone. The basal levels of cAMP in the presence of 200 μM Ro 20-1724 were 4.09 ± 0.20 pmol/10^6 cells. Forskolin increased the cAMP levels to 61.44 ± 2.78 pmol/10^6 cells. Shown are the results of one of three independent experiments.

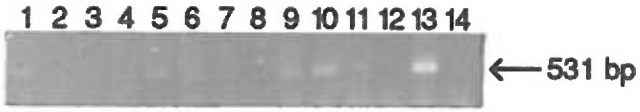

1 2 3 4 5 6 7 8 9 10 11 12 13 14
531 bp

Fig.6 Tissue distribution of R226 transcripts examined by RT-PCR. Lanes 1. striatum, 2. hippocampus, 3. hypothalamus, 4. pituitary, 5. cortex, 6. olfactory bulb, 7. cerebellum, 8. heart, 9. lung, 10. kidney, 11. liver, 12. adipose tissue, 13. testis, 14. negative control.

Acknowledgements: We thank L.Vallar and C.Bouvier for suggestions on cAMP assays. Research was supported by NIH grants MH45614 and MH48728 to O.C.; HL17670 and HL35134 to G.L.S. Q.-Y. Z. was supported by Glaxo Group Research Limited and Tartar fellowship. M.E.O. was supported by a NIH National Research Service Award (F32GM13713).

References

1. Stiles, G. L. (1986) *Trends Pharmacol. Sci.* **7**, 486-490.
2. Ramkumar, V., Pierson, G. & Stiles, G. L. (1988) *Prog. Drug Res.* **32**, 195-247.
3. Williams, M. (1987) *Ann. Rev. Pharmacol. Toxicol.* **27**, 315-345.
4. Fredholm, B. & Dunwiddie, T. V. (1988) *Trends Pharmacol. Sci.* **9**, 130-134.
5. Sebastiao, A. M. & Riberio, J. A. (1990) *Br. J. Pharmacol.* **100**, 55-62.
6. Stiles, G. L. (1990) *Clin. Res.* **38**, 10-18.
7. Nakahata, N., Abe, M. T., Matsuoka, I., Uno. T. & Nakanishi, H. (1991) *J. Neurochem.* **57**, 963-969.
8. Nakata, H. (1989) *J. Biol. Chem.* **264**, 16545-16551.
9. Olah, M. E., Jacobson, K. A. & Stiles, G. L. (1990) *Arch. Biochem. Biophys.* **283**, 440-446.
10. Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M., Dumont, J. E. & Vassart, G. (1989) *Science* **244**, 569-572.
11. Maenhaut, C., Van Sande, J., Libert, F., Abramowicz, M., Parmentier, M., Vanderhaegen, J. J., Dumont, J. E., Vassart, G. & Schiffmann, S. (1990) *Biochem. Biophys. Res. Commun.* **173**, 1169-1178.
12. Reppert. S. M., Weaver, D. R., Stehle, J. H. & Rivkees, S. A. (1991) *Mol. Endocrin.* **5**, 1037-1048.
13. Libert, F., Schiffmann, S. N., Lefort, A., Parmentier, M., Gerard, C., Dumont, J. E., Vanderhaeghen, J. J. & Vassart, G. (1991) *EMBO J.* **10**, 1677-1682.
14. Mahan, J. C., McVittie, L. D., Smyk-Randall, E. M., Nakata, H., Monsma, F. J., Jr., Gerfen, C. R. & Sibley, D. R. (1991) *Molec. Pharmacol.* **40**, 1-7.
15. Lin, W., Marcucci, Rabin, R. A. & Roth, J. A. (1991) *J. Biol. Chem.* **266**, 14457-14463.
16. Ribeiro, J. A. & Sebastiao, A. M. (1986) *Prog. Neurobiol.* **26**, 179-209.

17. Oliveira, J. C., Sebastiao, A. M. & Riberio, J. A. (1991) *J. Neurochem.* **57**, 1165-1171.
18. Ali, H., Cunha-Melo. J. R., Saul, W. F. & Beaven, M. A. (1990) *J. Biol. Chem.* **265**, 745-753.
19. Olah, M. E., Ren, H., Ostrowski, J., Jacobson, K. A. and Stiles, G. L. Submitted.
20. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1990) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. Zhou, Q.-Y., Grandy, D. K., Thambi, L., Kushner, J. A., Van Tol, H.H.M., Cone, R., Pribnow, D., Salon, J., Bunzow, J. B. & Civelli, O. (1990) *Nature*, **347**, 76-80.
22. Sanger, F., Nicklen, S. D. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA.* **74**, 5463-5467.
23. Chen, C, & Okayama, H. (1987) *Molec. Cell Biol.* **7**, 2745-2752.
24. Ramkumar, V., Olah, M. E., Jacobson, K. A. and Stiles, G. L. (1991) *Mol. Pharmacol.* **40**, 639-647.
25. Brown, B. L., Albano, J. D., Ekins, R. P. & Sgherzi, A. M. (1971) *Biochem. J.* **171**, 561-562.
26. O'Dowd, B. F., Lefkowitz, R. J. & Caron, M. G. (1989) *Ann. Rev. Neurosci.* **12**, 67-83.
27. Kozak, M. (1984) *Nucleic Acids Res.* **12**, 857-872.
28. Civelli, O., Bunzow, J. R., Grandy, D. K., Zhou, Q.-Y. & Van Tol, H. H. M. M. (1991) *Eur. J. Pharmacol.-Molec. Pharmacol. Sect.* **207**, 277-286.
29. Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomu, H., Takeyama, Y. & Nishizuka, Y. (1985) *J. Biol. Chem.* **260**, 12492-12499.
30. Dohlman, H. G., Caron, M. G. & Lefkowitz, R. J. (1987) *Biochemistry* **26**, 2657-2664.
31. Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) *Annu. Rev. Biochem.* **56**, 567-613.
32. Hershey, A. O., Dykema, P. E. & Krause, J. E. (1991) *J. Biol. Chem.* **266**, 4366-4374.

33. Stiles, G. L., Daly, D. T. and Olsson, R. A. (1985) *J. Biol. Chem.* **260**, 10806-10811.
34. Boeynaems, J. M. and Pearson J. D. (1990) *Trends Pharmacol. Sci.* **11**, 34-37.
35. Nanoff, C., Jacobson, K. A. and Stiles, G. L. (1991) *Molec. Pharmacol.* **39**, 130-135.
36. Meyerhof, W., Muller-Brechlin, R. & Richter, D. (1991) *FEBS letter* **284**, 155-160.

Abbreviations used: AMP-PCP, adenyl(β,γ-methylene)-diphosphonate; APNEA, N^6-2-(4-amino-3-iodo-phenyl)ethyladenosine; CGS21680, 2-(4-(2-carboxyethyl)phenylethylamino-5'-N-ethylcarboxamidoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; NECA, N-ethyladenosine-5'-uronic acid; PAPA-APEC, 2-[4-(-{2-[(4-aminophenyl)methylcarbonylamino]ethylaminocarbonyl}ethyl)phenyl]ethylamino-5'-N-ethylcarboxamidoadenosine; R/S-PIA, R/S-N^6-phenyl-2-propyladenosine; XAC, 8-{4-[([{(2-aminoethyl)amino}carbonyl]methyl)oxy]phenyl-1,3-dipropyl-xanthine.

DISCUSSION

A. Methodology

Basically there are three strategies to isolate the genes or cDNAs for G protein-coupled receptors.

1). Traditional approach: involves the purification of receptor protein. Once the receptor is purified to its homogeneity, peptide sequences can be obtained and then based on the peptide sequences, oligonucleotide probe(s) can be designed for genomic or cDNA library screening. The β2, α2 and α1 adrenergic receptors, M1 and M2 muscarinic receptors , LH-CG receptor and CCK-A receptor were cloned by this way (1-7). The disadvantage of this strategy is that most G protein-coupled receptors are rare membrane proteins and it is extremely difficult to purify them to their homogeneity.

2). Expression approach: involves the functional expression of G protein-coupled receptors in Xenopus oocytes or in eucaryotic cells. The oocyte expression approach involves injecting *in vitro* synthesized RNA from cDNA pools into oocytes and subsequently measuring response of oocyte membrane potential to perfused receptor ligands. Receptors that couple to PI turnover including receptors for substance K , 5HT1c, PAF, endothelin, TRH, thrombin, and metabotropic glutamate receptor have been successfully cloned by this

approach (8-14). Another expression approach involves the transfection of cDNA pools into eucaryotic cells and subsequently detection of the positive cDNA pool by radio-labeled (usually [^{125}I]-labelled) ligands. Many different receptor cDNAs including NGF receptor, TGF-β type II receptor, tumor necrosis factor receptor, and receptors for the interleukins as well as the noradrenaline transporter have been successfully isolated by this approach. Within the G protein-coupled receptor family, receptors for endothelin, angiotensin II, PTH, calcitonin, secretin, and IL-8 have been successfully cloned by this approach (15-20). The expression approach should be applicable to the cloning of every neuropeptide receptor, i.e. receptor clones for the opioid peptides.

3). Homology approach: derives from the existence of significant homology among the G protein-coupled receptors cloned by the two methods mentioned above. Technically the homology approach can be classified into two methods: the low stringency screening and PCR-based approach. At least a dozen genes or cDNAs for G protein-coupled receptors have been isolated by low stringency screening method (see references 20-29 of Introduction). Using a set or sets of degenerate oligonucleotides based on conserved regions of transmembrane II, III, VI and VII, several members of G protein-coupled receptors have been successfully cloned by the PCR-based homology approach including somatostatin receptors, A1, A2

and A3 adenosine receptor, 5HT1d, neuropeptide Y receptor, TSH receptor, members of odorant receptor family, and the dopamine D1 receptor (21-28).

The PCR-based homology approach used for the cloning of the dopamine D_1 receptors exemplifies the advantages and disadvantages associated with the homology approach. The major advantage associated with homology approach is that it does not require extensive knowledge of a particular receptor structure or function to allow for its cloning. The obvious disadvantage of homology approach is that it is difficult to direct this strategy to the cloning of one particular receptor. The cloning of the D_1 dopamine receptor is probably the most directed protocol devised. It still required the sequencing of 36 different clones (28).

In comparing the PCR-based homology approach with low stringency screening, there appears to be two advantages: 1) It allows for a size-selection of the PCR products and thus for detection of predominant DNA species, especially if the receptor is enriched in a particular tissue; and 2) one can generate products in a much shorter time as dozens of PCR products can be sequenced. Moreover, since the primers are part of the transmembrane sequences, sequence analysis of the PCR products reveals rapidly whether they might encode potential new receptors. In addition, the PCR-based homology approach should be specially useful to clone receptor

subtypes.

As the number of reports presenting the sequence of G protein-coupled receptors increases, our knowledge of their evolutionary relationships based on their homology increases. The accumulation of almost a hundred G protein-coupled receptor sequences so far should help to direct the homology approach to the cloning of specific receptors, which could partially overcome the disadvantage of the homology approach. Moreover, the identification of orphan receptors by homology approach for which the natural ligands are unknown is of great potential value. The tissue distribution of orphan receptors by *in situ* hybridization should be of potential importance for mapping new signaling pathways for neuropeptides and neurotransmitters. Finally, an orphan receptor clone might be useful for isolating its endogenous ligand, such as the cannabinoid receptor.

B. Dopamine D1 receptor

a. Cloning

To clone the dopamine D1 receptor, we took advantage of the PCR-based approach. This approach consists of synthezing two degenerate oligonucleotides corresponding to two highly conserved regions among amine receptors. The two oligonucleotides were used as primers in a PCR reaction for selective amplification of cDNAs. The cDNA mixture used for

the D1 receptor cloning was synthesized from rat striatal mRNA. The resulting PCR products were subcloned and several were chosen for sequencing at random until one of interest was found. To direct the PCR approach toward the specific cloning of D1 receptor, we added another technical step. As it is known that Gs-coupled catecholamine receptors have putative third cytoplasmic loop of 52-78 amino acids, PCR products were size-fractionated and products in the expected range were sequenced (28). Of the 24 PCR products, seven encoded potential G protein-coupled receptors, one of which was later shown to be the dopamine D1 receptor.

The PCR product of interest (R213) was used to screen a rat striatal cDNA library, from which partial cDNA clones were obtained. These partial clones were subsequently used to screen a human genomic library, since most catecholamine receptor genes lack introns in their coding regions (29). That enabled us to isolate a full-length clone. As expected, the gene for the human D1 receptor lacks introns at least within the coding region.

b. Primary sequence and structure model

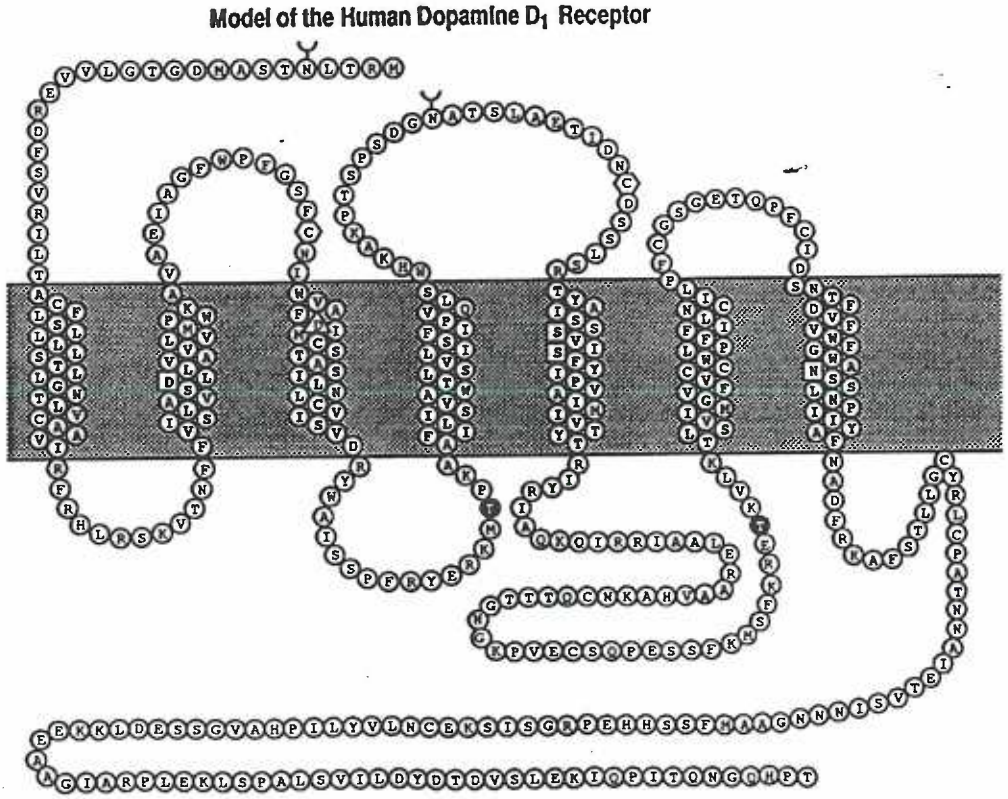
The translation start site for the human D1 receptor is somewhat ambiguous as there are two possible initiation methionines. By assuming the D1 receptor is N-glycosylated at its N-terminus like most of G protein-coupled receptor family members, we speculate the translation starts at the first MET

residue. Thus the human D1 receptor would be an 446 amino acids peptide with relative molecular weight of 49 KD. This calculated molecular weight is similar to the reported value of the deglycosylated form of the dopamine D1 receptor as determined by SDS-PAGE (30). There are two potential sites for N-linked glycosylation with one in the N-terminus and the other in the second extracellular loop. Previous experiments on protein preparations had indicated that the D1 receptor is a glycoprotein (30).

Hydrophobicity analysis of D1 receptor revealed seven stretches of hydrophobic amino acids that could represent transmembrane domains. Asp103 in the third transmembrane domain of D1 receptor possibly acts as counterion for the positive charge of dopamine. Two serines (residues 199 and 202) in the fifth transmembrane domain of the D1 receptor may form two hydrogen bonds with the catechol moiety of dopamine. Two Cys (residues 96 and 186) might form a disulfide bond which could be important for ligand binding (31). The conserved Cys in the C-terminus might be palmitolyated serving to anchor the receptor to the membrane (32). In addition, there are two consensus sequences (S/T X X R/K) for cAMP-dependent protein kinase phosphorylation and many serines and threonines in the cytoplasmic loops and the relatively long C-terminus could be potential phosphorylation sites for protein kinase C or receptor kinases (33). Interestingly, potential phosphorylation sites for casein

kinase II (S/T X X E/D) or cdc 2 kinase (S/T P X K/R) are also found in the D1 receptor (33). Tyrosine residues in the cytoplasmic loops and C-terminus of D1 receptor could be phosphorylation targets as well (34). Thus D1 receptor functions could be subjected to widespread regulation by phosphorylation mechanisms.

From the studies done on the β2-adrenergic receptor, is has been clear that the ligand binding site is located in the hydrophobic core while G protein interaction involves the cytoplasmic loops, especially the third cytoplasmic loop (31,35,36). Based on these results, we propose the following model for the dopamine D1 receptor (Fig.1). The D1 receptor peptide spans the membrane seven times. Its amino terminus is believed to be extracellular side of the cell while the carboxyl terminus protrudes inside the cell.



c. Functional characterization

Expression of the cloned human D1 receptor in COS-7, GH4 and Ltk^- cells have confirmed the pharmacological identity of this receptor subtype. Saturable and high-affinity binding of [^{3}H]SCH23390, a D1 receptor-selective antagonist, was demonstrated using transfected cell membranes. The [^{3}H]SCH23390 binding was displaced by dopamine receptor ligands with a potency order of SCH23390 > (+) butaclamol > flupenthixol >> haloperidol. This pharmacological profile explicitly identifies the binding site as that of a dopamine D1 receptor.

Importantly, the cloned human D1 receptor was also shown to mediate the stimulation of adenylate cyclase with a pharmacology identical to that seen in endogenous receptor expression tissue systems. Recently we and collaborators have studied the signaling of the cloned human D1 receptor in more detail with stably transfected GH4 and Ltk^- cells. We found that in addition to cAMP elevation, the D1 receptor is able to mobilize Ca^{++} probably in a cAMP-dependent manner (37). If this observation can also apply to the *in vivo* situation it indicates that a single D1 receptor is enough to stimulate adenylate cyclase and mobilize Ca^{++} as well (38).

d. Evolution

Comparison of the deduced amino acid sequence of D1 receptor with that of other catecholamine receptors shows that the greatest similarity exist in the putative transmembrane domains, where the amino-acid identities are: 44% with human D2 and 41-43% with human adrenergic receptor genes. Surprisingly, the overall degree of identity between D1 and D2 receptors is about the same as between D1 and any adrenergic receptor.

Sequence comparison revealed that the rat D1 receptor is about 86% identical to the human D1 at the nucleotide level and 92% identical at the amino acid level (28). The highest sequence similarity between rat and human D1 receptors was found in the putative transmembrane domains. Of the 41 amino acid residues different between rat and human D1 receptors, only four (9.8%) were found in the transmembrane domains. After correction for the numbers of residues and nucleotides in the transmembrane domains and in the loops, these represent significantly statistical difference (amino acid level: $x^2=14.29$, $p<0.001$, $df=1$; nucleotide level: $x^2=4.27$; $p<0.05$, $df=1$). This indicates that amino acid and nucleotide changes do not occur randomly and that there are fewer changes in the transmembrane domains than in the loops. This observation agrees with the conclusion that the ligand binding sites of G-protein coupled receptors reside in the transmembrane domains (36). In addition, most of the

nucleotide changes (140 out of 184) were found at the third codon position.

We have also isolated a partial mouse clone encoding D1 receptor. Based on the partial mouse sequence available (appendices), the mouse D1 gene sequence is almost identical to that of the rat gene, with 98% amino acid and 95% nucleic acid homology. The mouse D1 is 88% identical to human D1 at nucleotide level and 93% at amino acid level.

e. Promoter region

Primer extension studies have identified the putative transcriptional initiation site of the rat D1 receptor gene to be a cytidine, located 864 base pairs upstream the translation start site (Manuscript # 2 of this thesis). Analysis of 5'-flanking sequence of the rat D1 receptor gene revealed several structural features typical of a housekeeping gene including the absence of canonical TATA and CAAT boxes, a high G+C content and multiple binding sites for the transcription factor Sp1 (38). However, the D1 gene is expressed in a tissue-specific manner (see below). Many tissue-specific genes including some expressed only in neural cells, have similar housekeeping-type promoters: the NGF receptor gene, the Goα gene, the synapsin I gene and the protooncogene Pim-I (38).

Information contained within the 735 base pairs of 5'-

flanking sequence of the rat D1 gene appears to be sufficient to confer its cell-specific expression. This is demonstrated by transfection studies with the D1 gene 5' flanking sequence and CAT gene fusion constructs showing that CAT genes were active in neuroblastoma NS20Y cells that express D1 receptor endogenously but inactive in C6 and 293 cells that do not express the D1 receptor. Transfection studies have also shown that the D1 gene promoter responds to cAMP induction suggesting the existence of an autoregulation mechanism by which the stimulation of D1 receptor exerts a positive feedback on its own gene expression.

f. Tissue distribution

The tissue distribution of mRNA for the D1 receptor has been determined by Northern blot hybridization and *in situ* hybridization (28,39,40). In general, the localization of D1 receptor mRNA correlates well with regional distribution of D1 receptor by binding assays or autoradiography including the areas of highest expression: the caudate-putamen, nucleus accumbens, and olfactory tubercle. D1 receptor mRNA is also observed in the cereberal cortex, limbic area, hypothalamus and thalamus. Marked discrepancies between D1 receptor binding and messenger RNA were observed in other brain regions including the entopeduncular and subthalamic nuclei, substantia nigra pars reticulata, hippocampus and cerebellum (39). Much of the discordance between the distribution of receptor protein and mRNA is likely due to the differential

localization of D1 receptor messenger RNA in cell bodies and receptor binding sites which may remain localized in cell bodies or transported to efferent projections. Within the caudate-putamen, about 50% of the medium-sized neurons exhibit labeling of D1 mRNA, although a small number of the large-sized neurons may also express a low level (39). Preliminary evidence indicates that the majority of the medium-sized neurons that express D1 receptor mRNA belong to the striatonigral projection system and also express substance P (41).

An interesting and enigmatic finding to emerge over the past few years has been the interaction between D1 and D2 dopamine receptors at the functional (behavioral) level (42,43). The interaction between the two receptors can be either synergistic (for typical motor behaviors) or opposing (for repetitive jaw movements). Such functional interaction are currently not well understood and could occur by many different mechanisms. One mechanism may involve D1 and D2 coexpression in the same neuron while a second one possibly involves local axon collaterals that interconnect neurons expressing different receptors and a third one involves interneurons. *In situ* hybridization studies revealed that colocalization of D1 and D2 receptor mRNA occurred in one third of all caudate-putamen neurons in rats (40): about half of cell containing D1 receptor mRNA also contained D2 receptor mRNA. The coexpression of D1 and D2 receptor in the

dopaminoceptive cells in the caudate-putamen supports that D1/D2 interaction can occur at the cellular level, although it does not necessarily exclude other mechanisms. This observation agrees with a recent report indicating a synergistic D1/D2 interaction on inhibition of Na^+/K^+ ATPase of medium spiny striatal neurons, which probably requires the coexpression of D1 and D2 receptors on the same neuron (44).

g. Gene structure and localization

The cloning of rat gene and full-length cDNA revealed the existence of a single intron in the 5' untranslated region of the D1 receptor mRNA (manuscript #2 of this thesis). This gene organization is similar to that of the muscarinic receptors (45).

Genomic southern blot hybridization of human DNA indicates that dopamine D1 receptor gene is a single copy. The human D1 receptor gene has been assigned to chromosome 5 by southern blot hybridization with DNAs from a rodent-human somatic cell hybrid panel (46). Fluorescent *in situ* hybridization of D1 gene to human metaphase chromosome refined the location of D1 gene to 5q35.1. It is of some evolutionary interest that two other human catecholamine receptors α1 and β2 adrenergic receptor genes, which are closely related to D1 receptor, are also located on chromosome 5 at q32-34 and q31-32, respectively (47). Tandem replication of an ancestor gene could be their evolutionary origins. Moreover, two RFLPs

associated with dopamine D1 receptor gene have been identified (46,48).

h. Significance and perspectives

The cloning and functional expression of the dopamine D1 receptor has provided a better understanding of its genetic structure and regulation. Northern blot and *in situ* hybridization of D1 receptor has given us a better picture of anatomical organization of dopaminergic neurotransmission in the CNS.

Andersen et al summarized the inadequacy of D1/D2 classification for dopamine receptor a few years ago (Tab 1). According to Andersen et al, up to nine different dopamine receptor identities may exist. Five different dopamine receptors have been cloned and identified using molecular biological techniques (28, 49-52) (Tab 2).

TABLE I. Properties of putative subtypes of dopamine receptor

Classical subtype	Anatomical location	Pharmacology: agonists	Pharmacology: antagonists	Transmembrane signalling
D_1	striatum, frontal cortex, nucleus accumbens	SKF75670 > SKF77434 > SKF38393 >> 5,6-ADTN ≃ 6,7-ADTN > dopamine	NO112 ≃ NO756 > fluperlapine ≃ clozapine ≃ SCH23390 ≃ *cis*-flupentixol > bulbocapnine >> sulpiride	↑ adenylyl cyclase
	amygdala, striatum, frontal cortex, nucleus accumbens	SKF75670 > SKF38393 ≃ SKF77434 >> dopamine ≃ 6,7-ADTN > 5,6-ADTN	SCH23390 ≃ NO112 ≃ NO756 ≃ *cis*-flupentixol >> clozapine > fluperlapine > bulbocapnine >>> sulpiride	? ↑ polyphospho-inositide hydrolysis
Peripheral D_1	mesenteric bed	fenoldopam > dopamine > SKF38393	SCH23390 > butaclamol ≃ bulbocapnine > sulpiride >> haloperidol	↑ adenylyl cyclase
	splenic artery, renal cortex	dopamine >> SKF38393 ≃ fenoldopam	SCH23390 > butaclamol > bulbocapnine > haloperidol >> sulpiride	↑ adenylyl cyclase
	renal cortex	no conclusive data	SCH23390 ≃ sulpiride	? ↑ phospholipase C
D_2	striatum, limbic areas, retina	no conclusive data	spiperone > haloperidol > sulpiride ≃ clozapine	↓ adenylyl cyclase open K^+ channels
	striatum, pituitary, retina	no conclusive data	spiperone > haloperidol >> sulpiride ≃ clozapine	? ↓ phospho-lipase C ? ↓ Ca^{2+} channels ? ↓ polyphospho-inositide hydrolysis
Peripheral D_2	heart	DP-5,6-ADTN > DP-6,7-ADTN > dopamine >> SKF38393 ≃ fenoldopam	haloperidol > *cis*-flupentixol > sulpiride >> SCH23390	↓ adenylyl cyclase
	mesenteric artery	DP-5,6-ADTN > dopamine ≃ DP-6,7-ADTN > fenoldopam >> SKF38393	haloperidol ≃ sulpiride >> *cis*-flupentixol ≃ SCH23390	no data

TABLE 2. Comparison of dopamine (DA) receptor subtypes

Receptor isoforms	D_1	D_2 *(short)*	D_2 *(long)*	D_3	D_4	D_5
Brain regions enriched	C/P	C/P	C/P	OT	FCX	OT,NA
	OT	OT	OT	NA	AMG	FCX
	NA	NA	NA	IC	Midbrain	C/P
Posterior pituitary	Absent	Present	Present	Absent	—	—
Nigral DA cells	No	Yes	Yes	Yes	—	—
GTP regulation	Yes	Yes	Yes	No (?)	Yes	—
Adenylyl cyclase	Stim.	Inhib.	inhib.	No effect[a]	—	Stim.
Affinity for dopamine	Micromolar	High nM	High nM	Low nM	Low nM	High nM
Affinity for clozapine	Low	Low	Low	Low	High	Low
Butyrophenone potency	Micromolar	Subnanomolar	Subnanomolar	Nanomolar	Subnanomolar	Micromolar
Amino acids						
Rat	446	415	444	446	—	—
Human	446	414	443	—	387	477
Amino acid sequence homology in transmembrane domains Versus D_2 (long)						
	44%	100%	100%	75%	53%	47%

C/P, caudate/putamen; OT, olfactory tubercle; NA, nucleus accumbens; IC, islands of Calleja; FCX, frontal cortex; AMG amygdala.
[a]In CHO cells.

D2 receptor family

The dopamine D2 receptor was the first dopamine receptor to be cloned by using a β2-adrenergic receptor cDNA to probe a rat genomic library (52a). Shortly after the initial cloning of the dopamine D2 receptor, it was demonstrated that this receptor exists in two protein forms that differ in length of 29 amino acids and derived from the same gene by alternative RNA splicing (52b). No functional difference has been observed between these two isoforms (52c). The D3 and D4 receptors were identified by probes derived from the D2 receptor sequence (51,52). The D3 receptor is 52% homologous to the D2 receptor overall, and 75% homologous in TM regions. The overall homology of the D4 receptor to the D2 and D3 receptors is 41% and 39% respectively, but the homology increases to about 56% for both receptors within the TM regions. When expressed in eucaryotic cells, the pharmacology of D2, D3 and D4 receptors is similar but not identical to one another. The most notably differences are: 1). The D3 receptor has 20-fold higher affinity for dopamine than the D2 and D4 receptors; 2). The D4 receptor has much high affinity for the antipsychotic drug clozapine than the D2 and D3 receptors. The distribution of D2, D3 and D4 receptors are different when examined by Northern blot hybridization and in situ histochemistry. The area of highest expression of the D2 receptor correspond to major dopaminergic projection areas

including the caudate putamen, nucleus accumbens and olfactory tubercle (52d). The D2 receptor is also expressed in the pituitary, substantia nigra par compacta and ventral tegmental areas (52d). As with the two isoforms of the D2 receptor, the larger form appears to be expressed predominantly in all regions, although the exact ratio of the two isoform can vary significantly (52c). The D3 and D4 receptors are expresssed at least one order of magnitude lower than that of the D2 receptor and are more narrowly distributed(51,52). The D3 receptor is expressed predominantly in limbic areas including olfactory tubercle, nucleus accumbens and hypothalamus (51). The D4 receptor is expressed predominantly in frontal cortex, midbrain, amygdala and medulla (52).

D1 receptor family

Very recently, a novel D1 subtype (D1b, or termed D5) receptor gene has been isolated (49,50). Human D1b and D1 share 50% overall sequence identity and 82% identity in the transmembrane domains. D1b and D1 receptors are almost pharmacologically identical, although D1b appears to have higher affinity for dopamine than D1 receptor (49,50). The D1b receptor is primarily expressed within the limbic regions of the brain and may also be expressed in the kidney (49,50). The cloning of D1b gene, on one hand, suggests that previous studies on "D1" receptors represent the combined participation of both D1 and D1b receptor and yet

unidentified D1 receptor subtypes. Thus, earlier biochemical, behavioral and electrophysiological studies need to be reevaluated for the effects of drugs on D1 or D1a receptor. On the other hand, cloning of new dopamine receptors(D1b, D3, and D4) has made the screening of more selective dopamine ligands feasible (51,52). Clinically it seem that long-term treatment of Parkinson's disease with L-dopa in many cases leads to the development of psychosis due to the excess dopamine receptor activation in limbic and cortical areas. Similarly, treatment of schizophrenia with neuroleptics often produces adverse side-effects such as parkinsonism and tardive dyskinesia due to the blocking of nigrostriatal dopamine receptor (53). Thus, the development of more specific dopamine agonists and antagonists that can be used in the treatment of Parkinson's disease and schizophrenia is clinically very important. Cloning and functional expression of dopamine receptor genes or cDNAs would make such screening for better dopamine drugs more feasible.

There are a number of experiments worth pursuiting using the cloned dopamine receptor genes or cDNAs:

1). Screen for more specific dopamine drugs. For example, the use of clozapine, which lacks most of the common side effects of neuroleptics, is limited because of the risk of agranulocytosis. The isolation and expression of the D4 receptor gene may help in the design of improved clozapine analogues.

2). Generate specific antibodies against individual dopamine receptors. These antibodies will be very useful tools for studying the dopamine receptors. For example, these antibodies may be used to study the topography of dopamine receptor molecules and to study the receptor protein distribution by in situ histochemistry.

3). Map the ligand binding sites of dopamine receptors by site-directed mutagenesis, deletion and chimeric dopamine receptors.

4). Overexpress individual receptors in eucaryotic cells or bacteria. Receptors may then be purified from overexpressed sources, and purified receptor protein can then be reconstituted into liposome or even be crystallized. Dopamine receptor protein crystals can then be used for tertiary structure determination. The knowledge of the tertiary structure of dopamine receptors and their ligand binding sites will help to design better dopamine drugs.

5). Disrupt the individual dopamine receptor gene by homologous recombination in mouse embryonic stem (ES) cells and subsequent production of transgenic mice lacking the encoded receptors by blastocyst injection of the ES clones. Mice lacking one of the five dopamine receptor gene will provide excellent animal models to study the biological functions of individual dopamine receptors.

C. A3 adenosine receptor

Our results show that R226 encodes a novel adenosine receptor subtype. R226 possesses several structural features that place it in the adenosine subfamily of G protein-coupled receptors. First, the predicated amino acid sequence of R226 shows high homology with recently cloned A1AR and A2AR (54,55). The highest homology in the putative transmembrane domain is between R226 and canine A1AR (58%), and between R226 and canine A2AR (57%). Identities between R226 and other G protein-coupled receptors are less than 30%. This degree of homology in transmembrane regions is a little higher than that between D1 and D2 dopamine receptors, between $\alpha 1, \alpha 2$ and β adrenergic receptor subtypes (which in case is about 42%) but not higher enough to place it in a receptor subtype of either A1AR and A2AR (The sequence homology between three $\alpha 1$ adrenergic receptor genes, between three $\alpha 2$ adrenergic receptor genes, between three β adrenergic receptor genes, between two D1 dopamine receptor genes, and between two dopamine D2 receptor subtype is over 70% in each case). Second, there is not aspartic acid residue in the third transmembrane domain of A1AR, A2AR and R226. Furthermore, there is a N-linked glycosylation site found in the second extracellular loop of A1AR, A2AR and R226. But in contrast to A1AR and A2AR, which lack N-linked glycosylation site in N-terminus, R226 has two extra potential N-linked glycosylation sites in its short N-terminus.

Ligand binding studies confirm that R226 encodes a novel

adenosine receptor. CHO cells stably transfected with R226 specifically bind nonselective adenosine agonist [^{3}H]NECA and A1 agonist [^{125}I]APNEA but not to A1 selective antagonists [^{3}H]DPCPX and [^{3}H]XAC, or the A2 selective agonist ligands [^{3}H]CGS21680 and [^{125}I]PAPA-APEC. Extensive characterization with [^{125}I]APNEA showed that R226 bind [^{125}I]APNEA with affinity constant Kd of 17 nM, one order of magnitude lower than typical A1AR (56). The specific [^{125}I]APNEA binding could be inhibited by adenosine ligands with a potency order of R-PIA = NECA > S-PIA > adenosine > ATP =ADP. A1 selective antagonists XAC and DPCPX could not inhibit [^{125}I]APNEA binding. The inability of R226 to recognize DPCPX and differentiate R-PIA from NECA makes R226 totally different from A1AR (56). The inability of R226 to bind A2 selective ligands [^{3}H]CGS21680 and [^{125}I]PAPA-APEC also makes R226 different from A2AR. Thus, the ligand binding characteristics of R226 are different from the recently cloned A1AR and A2AR.

R-PIA and NECA causes a dose-dependent inhibition of forskolin-stimulated cAMP level, suggesting the adenosine receptor encoded by R226 is coupled to inhibition of adenylate cyclase. Agreeing with the binding results, NECA-stimulated inhibition of cAMP accumulation in transfected CHO cells could not be blocked by the A1AR antagonist DPCPX.

The tissue distribution of R226 messenger RNA examined by RT-PCR is markedly different from that of A1AR and A2AR.

Compared to the high expression of A1AR and A2AR in the CNS, R226 is only very modestly expressed there but exhibits a higher expression in the peripheral tissues, especially in the testis. The adenosine receptor in the testis has only been modestly characterized (57). Recently a receptor sequence tgpcr1 same as R226 but without any pharmacological characterization was reported (58). Northern blot analysis of tgpcr1 showed that the expression of tgpcr1 was only detected in the testis (58). The difference between the Northern analysis of tgpcr1 and our RT-PCR results of R226 could be due to much higher sensitivity of RT-PCR than northern analysis. They have also shown that the expression of tgpcr1 mRNA correlates with sexual maturation of rats, revealing highest levels during the second and third months (58). By *in situ* hybridization, the mRNA of tgpcr1 has been localized in spermatocytes and spermatids but not spermatogonia, Leydig or Sertoli cells (58). Thus A3 adenosine receptor could modulate the process of spermiogenesis.

The molecular cloning of A3 adenosine receptor subtype indicates that adenosine receptor subfamily contains additional members. The cloning of A3 adenosine receptor also provides a tool for the elucidation of the role of adenosine in the spermiogenesis. Moreover, the molecular cloning of distinct adenosine receptor subtypes will help define the signal transduction pathway activated by different adenosine receptor subtypes.

References

1. Dixon, R.A.F., Koblika, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., Frielle, T., Bolanowski, M., Bennett, C., Rands, E., Diehl, R., Mumford, R., Slater, E., Sigal, I., Caron, M., Lefkowitz, R., Strader, C. Cloning of the gene and cDNA for mammalian β-adrenergic receptor receptor and homology with rhodopsin. *Nature* **321**, 75-79, 1986.

2. Kobilka, B. K., Matsui, H., Kobilka, T.S., Yang-Feng, T. L., Francke, U., et al. Cloning, sequencing and expression of the gene coding for human platelet α2-receptor. *Science* **238**, 650-656, 1987.

3. Cotecchia, S., Schwinn, D. A., Randall, R.R., Lefkowitz, R. J., Caron, M. G., & Kobilka, B. K. Molecular cloning and expression of the cDNA for hamster alpha1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **85**, 7159-7163, 1988.

4. Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., et al. Cloning, sequencing and expression of the complementary DNA encoding the muscarinic acetylcholine receptor. *Nature* **323**, 411-416, 1986.

5. Kubo, T., Maeda, A., Sugimoto, K., Akiba, I., Mikami, A., et al. Primary structure of porcine cardic muscarinic acetylcholine receptor deduced from the cDNA sequence. *FEBS lett.* **209**, 367-372, 1986.

6. McFarland, K. C., Sprengel, R., Philips, H. S., Kohler, M., Rosemblit, N., Nikolics, K., Segaloff, D. & Seeburg, P. Lutropin-choriogonadotropin receptor: an usual member of the G protein-coupled receptor family. *Science* **245**, 494-499, 1989.

7. Wank, S. Harkins, R., Jensen, R., Shapira, H., Weerth, A. D. & Slattery, T. Purification, molecular cloning, functional expression of the cholecyctokinin receptor from rat pancreas. *Proc. Natl. Acad. Sci. USA* **89**, 3125-3129, 1992.

8. Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M., & Nakanishi, S. cDNA cloning of bovine substance K-receptor through oocyte expression system. *Nature* **329**, 836-838, 1987.

9. Julius, D., MacDermott, A. B., Axel, R. & Jessell, T. M. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. *Science* **241**, 558-564, 1988.

10. Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T. & Shimizu, T. Cloning by functional expression of plate-activating factor receptor from guinea-pig lung. *Nature* **349**, 342-346, 1991.

11. Arai, H., Hori, S., Aramori, I., Ohkubo, H. & Nakanishi, S. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* **348**, 730-732, 1990.

12. Straub, R. E., Frech, G. C., Joho., R. H. & Gershengorn, M. C. Expression cloning of cDNA encoding the mouse pituitary thyrotropin-releasing hormone receptor. *Proc. Natl. Acad. Sci. USA* **87**, 9514-9518, 1990.

13. Vu., T., Hung, D. T., Wheaton, V. I. & Coughlin, S. R. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057-1068, 1991.

14. Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R. & Nakanishi, S. Sequence and expression of a metabotropic

glutamate receptor. *Nature* **349**, 760-765, 1991.

15. Sakarai, T., Yanagissawa, M., Takuwa, Y., Miyazaki, S., Goto, K. & Masaki, T. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* **348**, 732-735, 1990.

16. Murphy, T. J., Alexander, R. W., Griendling, K. K., Runge, M. S. & Berstein, K. E. Isolation of a cDNA encoding the vascular type1 angiotensin II receptor. *Nature* **351**, 233-236, 1991.

17. Abou-Samra, A., Jupper, H., Force, T., Freeman, M. et al. Expression cloning of a common receptor for parathyroid hormone and parathroid hormone-related peptide from rat osteoblast-like cells. *Proc. Natl. Acad. Sci. USA* **89**, 2732-2736, 1992.

18. Lin, H. Y., Harris, T. L., Flannery, M. S.m Aruffo, A. Kaji, E. H., Gorn, Kolakowski, Jr., Lodish, H. F. & Goldring, S. R. Expression cloning of an adenylate cyclase-coupled calcitonin receptor. *Science* **254**, 1011-1024, 1991.

19. Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, T., Takahashi, K. & Nagata, S. Molecular cloning and expression of a cDNA encoding the secretin receptor. *EMBO J.* **10**, 1635-1641, 1991.

20. Murphy, P. & Tiffany, H. L. Cloning of cDNA encoding a functional human interleukin-8 receptor. *Science* **253**, 1280-1283, 1991.

21. Yamada, Y., Post, S. R., Wang, K., Tager, H. S., Bell, G. I. & Seino, S. Cloning and functional characterization of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract, and kidney. *Proc. Natl. Acad. Sci.*

USA. **89**, 251-255, 1992.

22. Maenhaut, C., Van Sande, J., Libert, F., Abramowicz, M., Parmentrier, M., Vanderhaegen, J. J., Dumont, J. E., Vassart, G. & Schiffmann, S. (1990) RDC8 codes for an adenosine receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* **173**, 1169-1178.

23. Mahan, J. C., McVittie, L. D., Smyk-Randall, E. M., Nakata, H., Monsma, F. J., Jr., Gerfen, C. R. & Sibley, D. R. (1991) Cloning and expression of an A1 adenosine receptor from rat brain. *Molec. Pharmacol.* **40**, 1-7.

24. Hamblin, M. W. & Metcalf, M. A. Primary structure and functional characterization of a human 5-HT1d serotonin receptor. *Molec. Pharmacol.* **40**, 143-148, 1991.

25. Rimland, J., Xin. W., Sweetnam, P., Saijoh, K., Nestler, E. J. & Duman, R. S. Sequence and expression of a neuropeptide Y receptor cDNA. *Molec. Pharmacol.* **40**, 869-875, 1991.

26. Parmentrier. M., Libert, F., Maehaut, C., Lefort, A., Gerard, C., Perret, J., Van Sande, J., Dumont, J. E. & Vassart, G. Molecular cloning of the thyrotropin receptor. *Science* **246**, 1620-1622, 1989.

27. Buck, L. & Axel, R. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* **65**, 175-187, 1991.

28. Qun-Yong Zhou, David K. Grandy, Lisa Thambi, Jake A. Kushner, Hubert H. M. VanTol, Roger Cone, David Pribnow, John Salon, James R. Bunzow & Olivier Civelli. Cloning and Expression of Human and Rat D1 Dopamine Receptors. *Nature* **347**, 76-80, 1990.

29. Dohlman, H. G., Thorner, J., Caron, M. G. & Lefkowitz, R. J. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**, 653-688, 1991.

30. Jarvie, K. R., Booth, G., Brown, E. M. & Niznik, H. B. Glycoprotein nature of dopamine D1 receptor in the brain and parathroid gland. *Molec. Pharmacol.* **31**, 566-574, 1989.

31. Dixon, R. A. F., I. S. Sigal, M. R. Candelore, R. B. Register, W. Scattergood, E. Rands & C. D. Strader. Structural features required for ligand binding to the β-adrenergic receptor. *EMBO J.* **6**, 3269-3275, 1987.

32. O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J. & Bouvier, M. Palmitoylation of the human β2-adrenergic receptor. *J. Bio. Chem.* **264**, 7564-7569, 1989.

33. Edelman, A. M., Blumenthal, D. k. & Krebs, E. G. Protein serine/threonine kinases. *Annu. Rev. Biochem.* **56**, 567-613, 1987.

34. Valiquette, M., Bonin, H., Hnatowich, M. Caron, M. G. Lefkowitz, R. J. & Bouvier, M. Involvement of tyrosine residues located in the carboxyl tail of the human β2-adrenergic receptor in agonist-induced down regulation of the receptor. *Proc. Natl. Acad. Sci. USA.* **87**, 5089-5093, 1990.

35. Strader, C. D., Sigal, I. S., Register, R. B., Candelore, M. R., Rands, E. & Dixon, R. A. F. Identification of residues required for ligand binding to the β-adrenergic receptor. *Proc. natl. Acad. Sci. USA.* **84**, 4384-4388, 1987.

36. O'Dowd, B. F. Hnatowich, M. Regan, J. W. Leader, W. M. Caron, M. G. & Lefkowitz, R. J. Site-directed mutagenesis of the cytoplasmic domains of the human β2-adrenergic receptor.

J. Bio. Chem. **263**, 15985-15992, 1988.

37. Yang F. Liu, Qun-Yong Zhou, Olivier Civelli & Paul Albert. Stimulation of the Cloned Human Dopamine D1 Receptor Induces a Cell-specific, Cholera Toxin Sensitive Calcium Mobilization. Submitted to Molecular Pharmacology.

38. Seghal, A., Patil, N. & Chao, M. A constitutive promoter directs expression of the NGF receptor gene. *Molec. Cell Biol.* **8**, 3160-3167, 1988.

39. Alfred Mansour, James. H. Meador-Woodruff, Qun-Yong Zhou, Olivier Civelli, Huda Akil & Stanley J. Watson. Comparison of D1 receptor binding and mRNA in the rat brain using receptor autoradiographic and *in situ* hybridization techniques. *Neuroscience* **45**, 359-371, 1991.

40. James. H. Meador-Woodruff, Alfred Mansour, Qun-Yong Zhou, James R. Bunzow, Olivier Civelli & Stanley J. Watson. Comparison of the distribution of D1 and D2 dopamine receptor mRNAs in the rat brain. *Neuropsychopharmacol.* **5**, 231-242, 1991.

41. Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z., Chase, T. Monsma, F. J. & Sibley, D. R. D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* **250**, 1429-1432, 1990.

42. Clark, D. & White, F. J. D1 receptor-the search for a function: a critical evaluation of the D1/D2 dopamine receptor classification and its functional implications. *Synapse* **1**, 347-348, 1987.

43. Wadditington, J. L. Functional interactions between D1 and D2 dopamine receptor system: their role in the regulation of psychomotor behavior, putative mechanisms and clinical

relevance. *J. Psychopharmacol.* **3**, 54-63, 1989.

44. Bertorello, A. M., Hopfield, J. F., Aperia, A. & Greengard, P. Inhibition by dopamine of Na+-K+ ATPase activity in neostriatal neurons through D1 and D@ dopamine receptor synergism. *Nature* **347**, 386-388, 1990.

45. Bonner, T. I., Buckley, N. J., Young, A. C. & Brann, M. R. Identification of a family of muscarinic receptor genes. *Science* **237**, 527-532, 1987.

46. David K. Grandy, Qun-Yong Zhou, Lee Allen, Ruth Litt, R. Ellen Magenis, Olivier Civelli & Michael Litt. A Human D1 Dopamine Receptor Gene Is Located on Chromosome 5 at q35.1 and Identifies an EcoRI RFLP. *American Journal of Human Genetics* **47**, 828-834, 1990.

47. Yang-Feng, T. L., Xue, F. Zhong, W., Cotecchia, S., Frielle, T. Caron, M. G. & Lefkowitz, R. J. Chromosomal organization of adrenergic receptor genes. *Proc. Natl. Acad. Sci. USA* **87**, 1516-1520, 1990.

48. M. Litt, M. Al-Dhalimy, Q. Zhou, D. Grandy & O. Civelli. A TaqI RFLP at the DRD1 locus. *Nucleic Acids Research* **19**, 3161, 1991.

49. David Grandy, Yuan Zhang, Claudia Bouvier, Qun-Yong Zhou, Robert A. Johnson, Lee Allen, Kari Buck, James R. Bunzow, John Salon & Olivier Civelli. Multiple Human D5 Dopamine Receptor Genes: a Functional Receptor and Two Pseudogenes. *Proc. Natl. Acad. Sci. USA.* **88**, 9175-9179, 1991.

50. Sunahara, R. K., Guan, H. C., o'Dowd, B. F., Seeman, P., Laurie, L. G., Ng, G., George, S. R., Torchinia, J., Van Tol, H. H. M. & Niznik, H. B. Cloning of the gene for a human D5 receptor with higher affinity for dopamine than D1. *Nature*

350, 614-619, 1991.

51. Sokoloff, P., Giros, B., Martres, M. P., Bouthenet, M. L. & Schwartz, J. C. Molecular cloning and characterization of a novel dopamine receptor D3 as a target for neuroleptics, *Nature* **347**, 146-151, 1990.

52. Van Tol, H. H. M., Bunzow, J. r., Guan, H. C., Sunahara, R. K., Seeman, P. Niznik, H. B. & Civelli, O. Cloning of a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature* **350**, 610-614, 1991.

52a. Bunzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M., Neve, K. A., & Civelli, O. Cloning and expression of a cDNA encoding the dopamine D2 receptor. *Nature* **336**, 783-787, 1988.

52b. Grandy, D. K., Marchionni, M. A., Makam, H. Stofko. R., E., Alfano, M., Frothingham, L., Fischer, J. B., Burke-Howie, K. J., Bunzow, J. R., Sever, A. C. & Civelli. O. Cloning of the cDNA and gene for a human D2 dopamine receptor. *Proc. Natl. Acad. Sci. USA* **86**, 29762-9766, 1989.

52c. Neve, K. A., Neve, R. L., Fidel, S., Janowsky, A. & Higgins, G. A. Increased abundance of alternatively spliced forms of D2 dopamine receptor mRNA after denervation. *Proc. Natl. Acad. Sci. USA* **88**, 2802-2806, 1991.

52d. Meador-Woodruff, J. H. Mansour, A., Bunzow, J. R., Van Tol, H. H. M., Watson, S. & Civelli, O. Distribution of D2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci. USA* **86**, 7625-7628, 1989.

53. Creese, I. Dopamine receptors. In *the receptors* , ed. Conn, P. M. (Acadamic press, New York), Vol. **4**, pp 171-212, 1986.

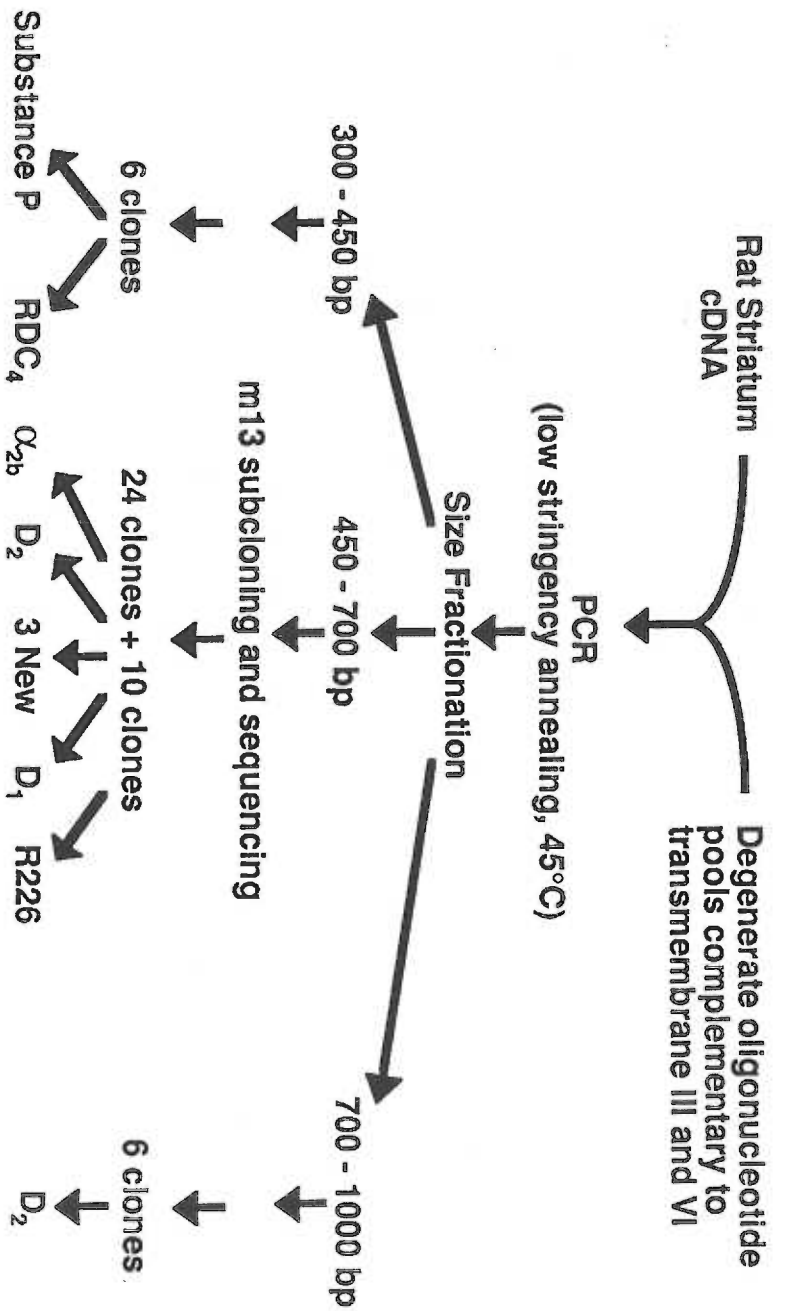
54. Libert, F., Schiffmann, S. N., Lefort, A., Parmentrier, M., Gerard, C., Dumont, J. E., Vanderhaeghen, J. J. & Vassart, G. The orphan receptor cDNA RDC7 encodes an A1 adenosine receptor. *EMBO J.* **10**, 1677-1682, 1991.

55. Maenhaut, C., Van Sande, J., Libert, F., Abramowicz, M., Parmentier, M., Vanderhaegen,J. J., Dumont, J. E. Vassart, G. & Schiffman, S. RDC8 codes for an A2 adenosine receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* **173**, 1169-1178, 1990.

56. Stiles, G. L., Daly, D. T. & Alsson, R. A. The A1 adenosine receptor. *J. Biol. Chem.* **260**, 10806-10811, 1985.

57. Conti, M., Boitanim, Demanno, D., Migliauo, S., Monaco, L. & Szymeczek, C. Characterization and function of adenosine receptors in the testis. *Annal NY Acad. Sci.* **564**, 39-47, 1989.

58. Meyerhof, W., Muller-Brechlin, B. & Richter, D. Molecular cloning of a novel putative G protein-coupled receptor expressed during rat spermiogenesis. *FEBS lett.* **284**, 155-160, 1991.


PCR - Homology Approach
Rat Striatum cDNA
Degenerate oligonucleotide pools complementary to transmembrane III and VI
PCR (low stringency annealing, 45°C)
Size Fractionation
300 - 450 bp
450 - 700 bp
700 - 1000 bp
m13 subcloning and sequencing
6 clones
24 clones + 10 clones
6 clones
Substance P
RDC4
α2b
D2
3 New
D1
R226
D2

NUCLEIC ACID SEQUENCE ALIGNMENT OF TWO UNKNOWN G PROTEIN-COUPLED RECEPTOR FRAGMENTS

```
        X       10        20        30        40        50        60        70
R21     CTGTGTGTCATTACGGTTGACGGCTACATTGCCGTGTGCCTCCCTGTGAAAGCTTTGGATTTCCGAACAC
        ||||||| ||| ||||||||||||| |  ||         ||   ||   |    |||  ||
R23     CTGTGTGTGATTGCGGTTGACGGCTACGTCACC----------CTCACCAATACCTCTCCCTCCTGGCAG
        X       10        20        30                  40        50        60

              80        90       100       110       120       130       140
        CTTTGAAAGCAAAGATCATCAACATCTGCATTTGGCTACTGGCATCATCTGTTGGTATATCAGCGATAGT
        |     ||||  ||      ||   |   ||   | ||| ||   ||    | || || ||  |
        CGCCACCAGCACCGAAT----ACGCAGCGCCGTGTGCGCAGGCGTCTGG-GTCCTCCTCTCCGCCATCAT
              70            80        90       100       110       120

                    150       160       170       180             190
        CCTT--------GGAGGCACCAAAGTCAGGGAAGATG-TGGATGTCATTGAA------TGCTCCTTGCTG
        ||          || || ||  |       || || |||||  |   ||       |||  ||| ||
        CCCACTGCCTGAGGTGGTACATATCC------AGCTGCTGGATGGCTCCGAGCCCATGTGCCTCTTCCTA
          130       140       150             160       170       180

          200       210       220       230       240       250       260
        TTTCCTGATGATGAATATTCCTGGTGGGACCTCTTCATGAAGATCTGTGTCTTCGTCTTTGCCTTTGTTA
           |||  |||    ||   |  |||| |||    ||   | |  |   | || | | ||| |
        GCACCTTTTGAAACGTACAGCGCCTGGCCCCTGGCAGTGGCCCTGTCGGCTACCATCCTGGCGTTCCTAC
                200       210       220       230       240       250

          270       280       290       300       310       320       330
        TCCCTGTCTTAATCATCATTGTCTGCTACACCCTGATGATCCTGCGCTTGAAGAGTGTCCGGCTCCTCTC
        | ||| |     |||||   || |   | | ||||     |||||
        TGCCTTTTCCTCTCATCGCAGTGTTTAATATCCTGTCAG-CCTGCC-----------------------
                270       280       290       300

          340       350       360       370       380       390
        GGGCTCTCGAGACAAGGACCGAAATCTCCGCCGGATCACCAAGCTGGTGCTGGTAGTGGTT---------
        ||||   |||  |||| | ||             ||   ||| || ||   |   |||
        -GGCTTCGGAGGCAAGGGCAGA-------------CAGAGAGCAGGCGCCACTGTCTGTTGATGTGGGC
            310       320                   330       340       350

                400       410       420       X
        -------GCAGTCTTCATCCTCTGCTGGCTGCCCCTACT
               || |||||  || ||||||||||||||||||
        TTACATAGCTGTCTTTGTCATCTGCTGGCTGCCCCTACT
                370       380       390       X
```

AMINO ACID SEQUENCE ALIGNMENT OF TWO UNKNOWN G PROTEIN-COUPLED RECEPTOR FRAGMENTS

```
     _III__                                  ___________IV___________
R21  LCVITVDRYIAVCLPVKALDFRTPLKAKIINICIWLLASSVGISAIVLGGTKVREDVDVIE
R23  LCVIAADRYVTLTNTSPSWQRHQHRIRRAVCAGVWVLLSAIIPLPEVVHIQLLDGSEPMCL

                                 _____________V____________
R21  CSLLFPDDEYWWDLFMKICVFVFAFVIPVLIIIVCYTLMILRLKSVRLLSGSREKDRNLRR
R23  FLAPF.ETYSAWALAVALSATILGFLLPFPLIAVFNILSACRLRRQGQTES.........R

          _______VI_________
R21  ITKLVLVVVAVFILCWLPY
R23  RHCLLMWAYAVFVICWLPY
```

AMINO ACID SEQUENCE ALIGNMENT OF HUMAN, RAT AND PARTIAL MOUSE DOPAMINE D1 RECEPTOR

```
hD1  MRTLNTSAMDGTGLVVERDFSVRILTACFLSLLILSTLLGNTLVCAAVIRFRHLRSKVTNFFVISLAVSDLLVA
rD1  M*AP...T..EA..PA.....F.................................................
mD1

hD1  VLVMPWKAVAEIAGFWPFGSFCNIWVAFDIMCSTASILNLCVISVDRYWAISSPFRYERKMTPKAAFILISVAW
rD1  ..........................................................Q...............
mD1                                            #.............Q........S.........

hD1  TLSVLISFIPVQLSWHKAKPTSPSDGNATSLAETI*DNCDSSLSRTYAISSSVISFYIPVAIMIVTYTRIYRIA
rD1  ......................W.L...F...E..ED....TR..........L...............S.....
mD1  ...........L.........W.L...F...EDAED....TR..........L...............S.....

hD1  QKQIRRIAALERAAVHAKNCQTTTGNGKPVECSQPESSFKMSFKRETKVLKTLSVIMGVFVCCWLPFFILNCIL
rD1  .......S...............A...Q....A.S.....................................S..MV
mD1  .......S...............T...N....S.S.....................................LS..MV

hD1  PFCGSGETQPFCIDSNTFDVFVWFGWANSSLNPIIYAFNADFRKAFSTLLGCYRLCPATNNAIETVSINNNGAA
rD1  .....E.........I.............................Q.............T..............V
mD1  .....E.........I.............................Q.............T..............V

hD1  MFSSHHEPRGSISKECNLVYLIPHAVGSSEDLKKEEAAGIARPLEKLSPALSVILDYDTDVSLEKIQPITQNGQ
rD1  V.............D.....................G...K.........................V.HS..
mD1  M.............D.................R...G..PK.........................HS..

hD1  HPT
rD1  .S.
mD1  .S.
```

Reprinted from Nature, Vol. 347, No. 6288, pp. 76–80, 6th September, 1990

Cloning and expression of human and rat D_1 dopamine receptors

Qun-Yong Zhou*†, David K. Grandy*, Lisa Thambi*‡, Jake A. Kushner*, Hubert H. M. Van Tol*§, Roger Cone*, David Pribnow||, John Salon*, James R. Bunzow* & Olivier Civelli*||¶

* Vollum Institute for Advanced Biomedical Research, † Department of Biochemistry and Molecular Biology, ‡ Department of Pharmacology and || Department of Cell Biology and Anatomy,
Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201, USA
§ Present address: Department of Pharmacology, Medical Sciences Building, University of Toronto, Toronto, Ontario M5S 1A8, Canada
¶ To whom correspondence should be addressed

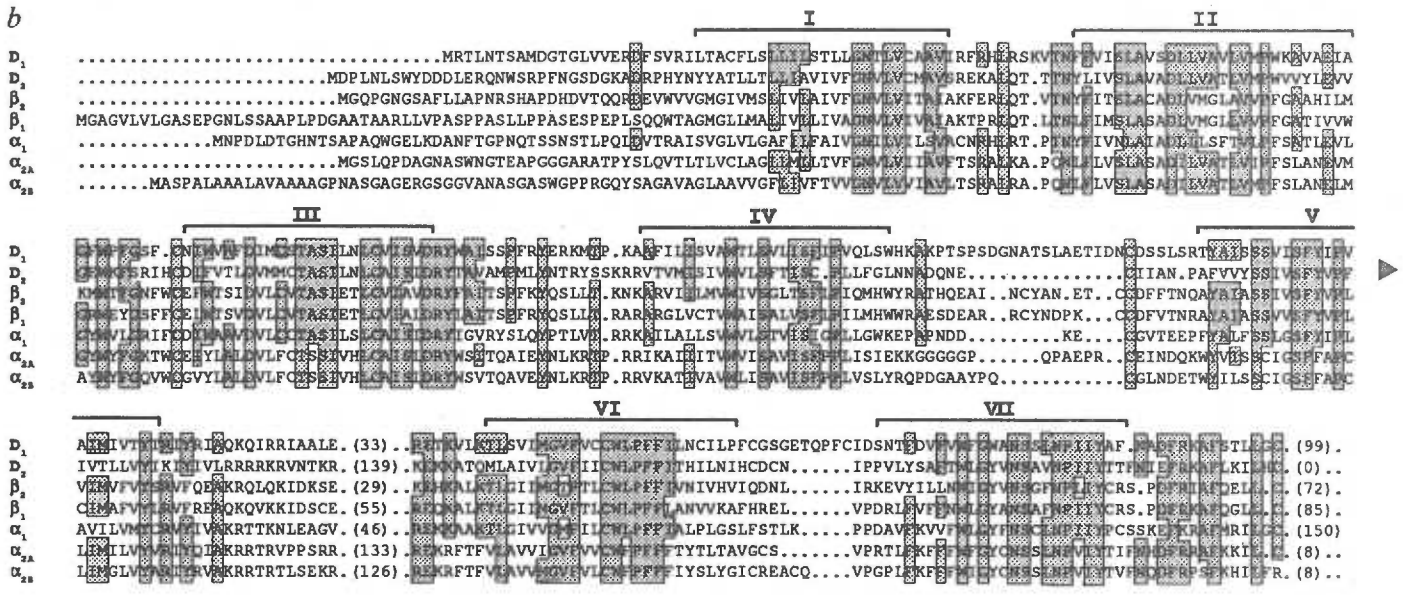
THE importance of the dopaminergic system in brain function has been emphasized by its association with neurological and psychiatric disorders such as Parkinson's disease and schizophrenia. On the basis of their biochemical and pharmacological characteristics, dopamine receptors are classified into D_1 and D_2 subtypes[1,2]. As the most abundant dopamine receptor in the central nervous system, D_1 receptors seem to mediate some behavioural responses[3], modulate activity of D_2 dopamine receptors[4,5], and regulate neuron growth and differentiation[6]. The D_2 dopamine receptor has been cloned by low-stringency screening[7]. We report here the cloning of human and rat D_1 dopamine receptors by applying an approach based on the polymerase chain reaction[8]. The cloned human D_1 dopamine receptor has been characterized on the basis of four criteria: the deduced amino-acid sequence, which reveals that it is a G protein-coupled receptor; the tissue distribution of its messenger RNA, which is compatible with that of the D_1 dopamine receptor; its pharmacological profile when transfected into COS-7 cells; and its ability to stimulate the accumulation of cyclic AMP in human 293 cells.

The D_1 and D_2 dopamine receptors are G protein-coupled receptors that stimulate and inhibit adenylyl cyclase, respectively[1,2]. Their structures should therefore contain the putative seven transmembrane domains common to G protein-coupled receptors[7]. The rat D_2 receptor was cloned by low-stringency screening using the hamster β_2-adrenergic receptor as a probe[7]. To clone the D_1 receptor, we applied a different approach. A set of degenerate oligonucleotide primers were designed based on the nucleotide sequences of known catecholamine receptors and some other G protein-coupled receptors[9,10]. Two primers, corresponding to putative receptor transmembrane domains III and VI, were used in a polymerase chain reaction (PCR)[8]. Rat striatum complementary DNA was chosen as the template because high levels of D_1 dopamine receptor have been found in this tissue[11]. Deletion studies have shown that the third cytoplasmic loop is crucial for G-protein coupling[12]. As the three cloned β-adrenergic receptors that couple to G_s proteins have putative third cytoplasmic loops of 52–78 amino acids[13], we hypothesized that the third cytoplasmic loop of the dopamine D_1 receptor might be in a similar size range. Therefore, the PCR products were size-fractionated and products ranging from 450–700 base pairs (bp) were subcloned into M13 and subjected to direct sequencing. Of 24 PCR products analysed, D_2 dopamine[7], α_{2B}-adrenergic[14] and five sequences representing potentially new G protein-coupled receptors were obtained. One of these clones, R213, had several interesting structural features. It had a higher degree of amino-acid similarity with known catecholamine receptors as compared with other G protein-coupled receptors; in the putative fifth transmembrane domain it contained two serine residues that were thought to be specific to receptors binding catecholamines[15]; and it had a putative third cytoplasmic loop similar in size and sequence to that of the β-adrenergic receptors[13].

The PCR-generated clone R213 was used as a probe to screen a rat striatum cDNA library. One positive clone was identified and sequenced. Although not full-length, it allowed us to describe most of the rat coding sequence (Fig. 1). As most catecholamine receptors lack introns in their coding regions[16] and as our preliminary human genomic analysis indicated the absence of introns in this gene (D. K. Grandy *et al.*, unpublished observations), we screened a human genomic library. Eight positive signals were obtained. One clone, HGR213-1, was further characterized and a 3.0-kilobase (kb) *Eco*RI–*Sac*I fragment spanning the whole coding region was subcloned and sequenced.

Figure 1 shows the nucleotide sequence of clone HGR213-1. The longest open reading frame codes for a 446-amino-acid protein (relative molecular mass 49,296 (M_r ~ 49K)). This M_r is similar to the reported value of the deglycosylated form of dopamine D_1 receptor as determined by SDS-PAGE[17]. Like most adrenergic receptors, but unlike the dopamine D_2 receptor[18], HGR213-1 has no intron in its coding sequence. There are two potential in-frame initiation sites. Considering the unique potential *N*-linked glycosylation site in the N-terminus, the initiation site shown in Fig. 1 is probably the one that is used. There is another potential *N*-linked glycosylation site in the second extracellular loop.

Hydrophobicity analysis of HGR213-1 revealed seven



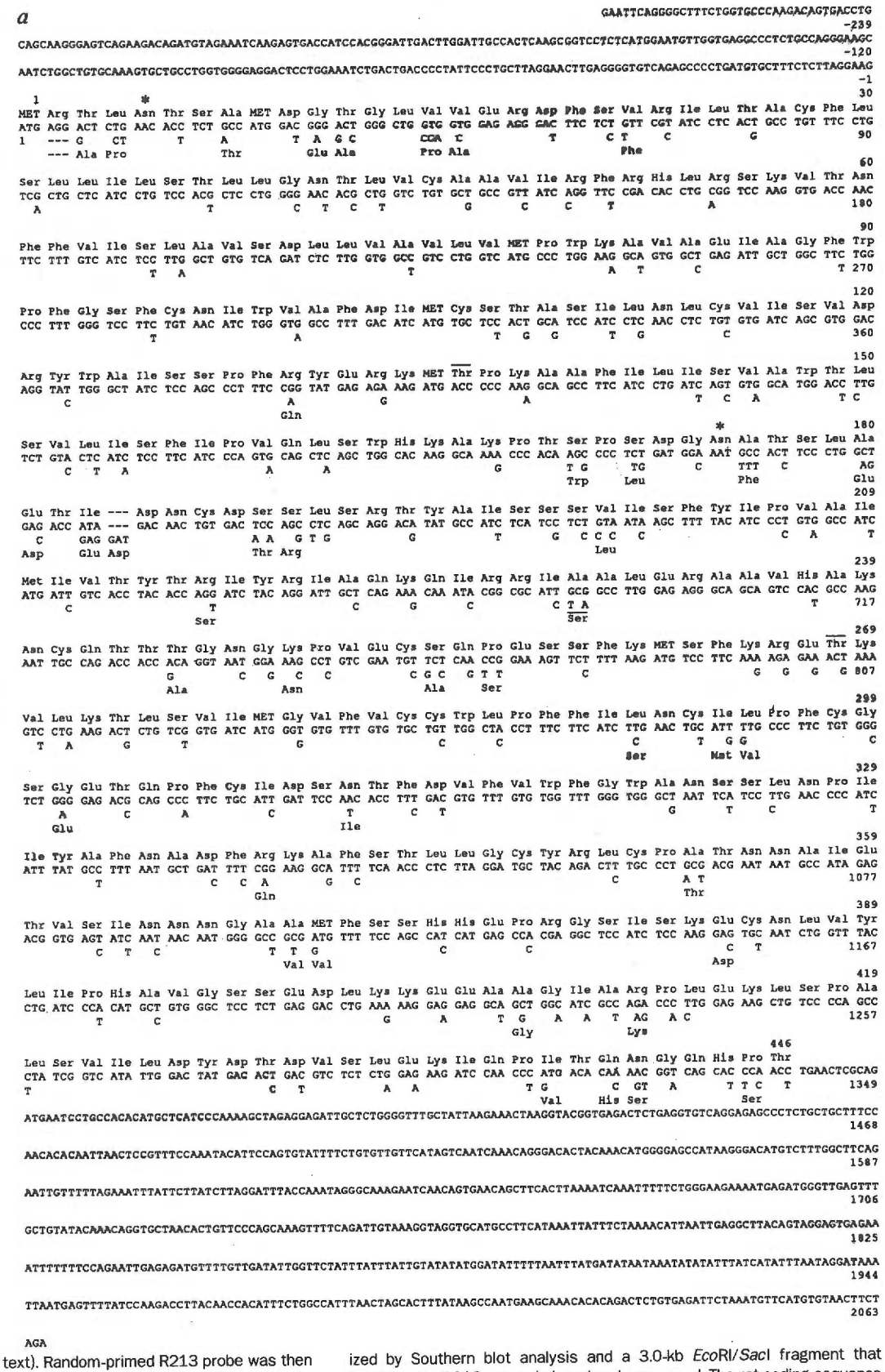


FIG. 1 *a*, Nucleotide and deduced amino-acid sequences of the human and rat D_1 dopamine receptors. Numbering begins with the first methionine of the open reading frame and is beneath the nucleotide sequence. The deduced amino-acid sequence of human D_1 receptor is shown above the nucleotide sequence. For rat D_1 sequences, the coding region and their differences with human D_1 are shown below the human D_1 sequences. Postulated *N*-glycosylation sites are indicated by asterisks and putative protein kinase A phosphorylation sites have a line above them. Dotted lines denote deletion. *b*, Alignment of the amino-acid sequence of the human D_1, human D_2, human β_2, human β_1, hamster α_1, human α_{2A} and human α_{2B} receptors. Shaded amino acids, residues that are conserved in at least three receptors and the D_1. The putative transmembrane domains are bracketed and labelled by Roman numerals. The number of residues in the variable third cytoplasmic loop and at the C terminus are in parentheses.

METHODS. Double-stranded cDNA was synthesized from rat striatum poly(A)$^+$ mRNA. Two degenerate oligonucleotides (III GAGTCGACCT-GTG(C/T)G(C/T)(C/G)AT(C/T)(A/G) CIIT (G/T) GAC (C/A)G(C/G)TAC, VI CAGAATTCAG(T/A) AGGGCAICCAGCAGAI(G/C)(G/A)(T/C)GAA) were designed based on the relatively conserved regions of receptor transmembrane domains III and VI. Rat striatum cDNA served as template in 30 cycles of PCR with 1 min of denaturation at 95 °C, 2 min of annealing at 45 °C, and 3 min of extension at 72 °C. The PCR products were double-digested with *Eco*RI and *Sal*I and the portion from 450–700 bp was extracted (GeneClean) and subcloned into M13mp18 and M13mp19. A total of 24 clones were sequenced. One clone, R213 (530 bp), was especially interesting (see text). Random-primed R213 probe was then used to screen in succession a rat striatum cDNA library, a human and a rat genomic library under stringent hybridization conditions[7]. One positive rat cDNA clone, five rat genomic clones and eight human genomic clones were isolated. One human genomic clone, HGR213-1, was further characterized by Southern blot analysis and a 3.0-kb *Eco*RI/*Sac*I fragment that hybridized to R213 was subcloned and sequenced. The rat coding sequence was obtained by sequencing of a partial rat cDNA clone and a rat genomic clone. Sequencing was by the Sanger dideoxy chain termination method using Sequenase (USB).

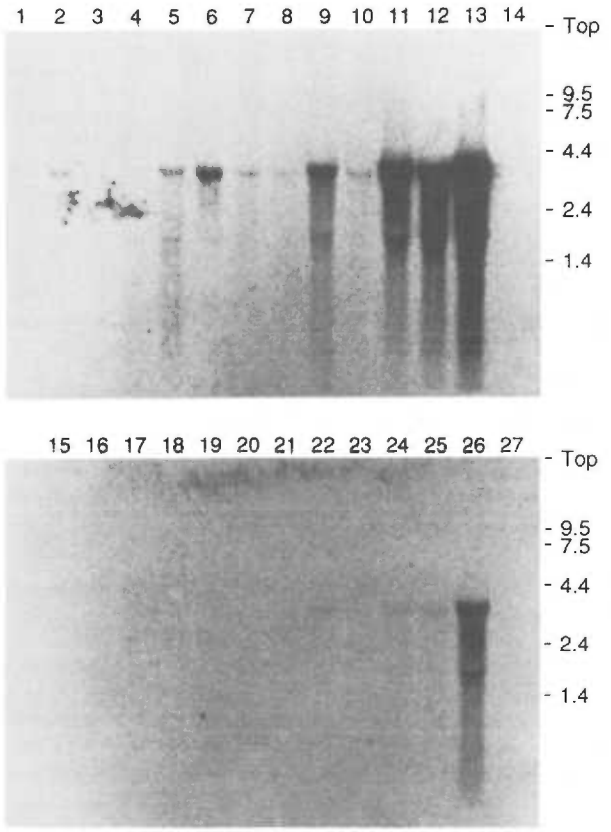


FIG. 2 Northern blot analysis of R213 transcripts in rat brain regions, pituitary and peripheral tissues. Northern blot analysis was performed as previously described, except random-primed R213 was used as hybridization probe[7]. Each lane contained 20 μg total RNA. Numbers on the right, RNA size markers (kb) (BRL). Lane 1, olfactory bulb; 2, hippocampus; 3, cerebellum; 4, posterior cortex; 5, anterior cortex; 6, thalamus; 7, hypothalamus; 8, medulla; 9, amygdala; 10, mesencephalon; 11, septum; 12, posterior basal ganglia; 13, anterior basal ganglia; 14, neurointermediate lobe of pituitary; 15, muscle; 16, ventricle; 17, atrium; 18, lung; 19, adrenal; 20, kidney; 21 liver; 22, pineal; 23, anterior lobe of pituitary; 24, hypothalamus; 25, mesencephalon; 26, posterior basal ganglia; 27, neurointermediate lobe of pituitary.

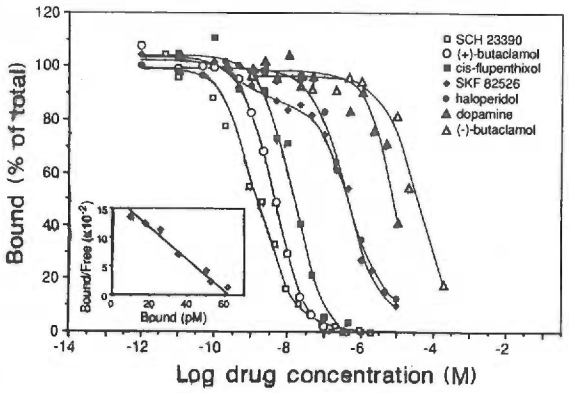


FIG. 3 Binding of [^{3}H]SCH23390 to membranes prepared from COS-7 cells transfected with HGR213-1. Representative curves show the competitive inhibition of [^{3}H]SCH23390 specific binding by different drugs. The inset shows a Scatchard transformation of saturation binding. The average inhibition constant, K_i, values from three independent experiments were: SCH23390, 0.4 nM; (+) butaclamol, 2.1 nM; *cis*-flupenthixol, 5.6 nM; haloperidol, 203 nM; dopamine, 2.3 μM; (−)butaclamol, 19 μM and SKF82526, 0.2 nM (high affinity) and 150 nM (low affinity). In the Scatchard plot shown, the dissociation constant K_d and the maximal number of binding sites, B_{max}, values for membranes prepared from transfected COS-7 cells were 0.3 nM and 2 pmol per mg protein, respectively.
METHODS. The 3.0-kb *Eco*RI–*Sac*I fragment of HGR213-1 was inserted between the unique *Hin*dIII and *Bam*HI sites of eukaryotic expression vector pBC12BI (ref. 27). A modified calcium phosphate method[29] was used for the transfection of COS-7 cells. About 45 μg plasmid DNA were used for each large 150 mm plate. At 48 h after transfection, cells were rinsed with TEM buffer (25 mM Tris buffer, pH 7.4, 6 mM $MgCl_2$, 1 mM EDTA) and scraped off plates. Membranes were prepared by homogenizing cells with a ConTorque homogenizer at 4 °C in TEM buffer. The homogenate was centrifuged at 800*g* for 10 min and the pellet again homogenized and centrifuged. Supernatants were pooled and centrifuged at 100,000*g* for 1 h. The pellet was then resuspended in TEM buffer at appropriate protein concentration and stored in small aliquots at −70 °C. Binding assays were performed in duplicate in 500 μl, containing 50 mM Tris buffer, pH 7.4, 0.9% NaCl, 0.025% ascorbic acid, 0.001% bovine serum albumin [^{3}H]SCH23390 (Amersham, 69 Ci mmol^{-1}) and tested drugs. In all competition binding assays, 0.7 nM [^{3}H]SCH23390 was inhibited by various concentrations of unlabelled drugs. Binding was initiated by the addition of membrane preparation (20–30 μg protein) and carried out at 30 °C for 1 h. Nonspecific binding was defined in the presence of 10 μM (+) Butaclamol. Samples were filtered through glass fibre filters (Schleicher and Schuell No. 32) and washed three times with 4 ml ice-cold 10 mM Tris buffer, pH 7.4. The radioactivity retained on the filter was counted using a Beckman LS6800 scintillation counter. The 50% inhibitory concentration values (IC_{50}) calculated from the curves were converted to K_i values as described[7]. Inhibition was fitted best by assuming the existence of only one class of binding site, except in the case of inhibition by the agonist SKF82526, which was best fitted by assuming the presence of two classes of binding sites. A LIGAND computer program was used for data analysis and curve fitting.

stretches of hydrophobic amino acids that could represent transmembrane domains (data not shown). Comparison of the deduced amino-acid sequence of HGR213-1 with that of other catecholamine receptors shows that the greatest similarity exists in the putative transmembrane domains, where the amino-acid identities are as follows: 44% with human D_2 (ref. 18); 42% with human β_2 (ref. 19); 43% with human β_1 (ref. 20); 41% with hamster α_1 (ref. 21); 42% with human α_{2A} (ref. 22) and 40% with human α_{2B} (ref. 14). The overall degree of identity between HGR213-1 and D_2 receptors is about the same as between HGR 213-1 and adrenergic receptors. Asp 79 and Asp 113 in the β_2-adrenergic receptor, which possibly act as counterions for the positively charged catecholamine[23], are present at corresponding positions in HGR213-1. Furthermore, the size and sequence of its third cytoplasmic loop and C terminus of HGR213-1 are similar to that of β-adrenergic receptors. This suggested to us that this new receptor might be coupled to G_s (ref. 12). But the absence of a potentially important glutamic acid residue[24], which is conserved in the third transmembrane domains of all three cloned β-adrenergic receptors[13] indicated that HGR213-1 probably was not a β-adrenergic-like receptor. On the basis of these structural features, we hypothesized that HGR213-1 could encode a dopamine D_1 receptor. In addition, there are two consensus sequences (residues 133–136, 265–268) for cAMP-dependent protein kinase phosphorylation[9], and the many serines and threonines in the cytoplasmic loops and the relatively long C terminus could be potential protein kinase C (ref. 25) or receptor kinase[26] phosphorylation sites.

As a step towards identifying HGR213-1, the tissue distribution of its transcript was examined by northern blot analysis. A messenger RNA of ~4 kb was found in many rat brain regions with the highest level of expression in the basal ganglia (Fig. 2). HGR213-1 mRNA was undetectable in the pituitary and in the peripheral tissues we tested. This pattern of HGR213-1 messenger distribution in the central nervous system and pituitary is consistent with that of the dopamine D_1 receptor as determined by autoradiography and binding studies[11].

To further investigate its identity, HGR213-1 was transiently expressed in eukaryotic cells. The 3.0-kb *Eco*RI–*Sac*I fragment of HGR213-1 was inserted into eukaryotic expression vector PBC12I (ref. 27) and transfected into monkey kidney COS-7 cells. As its structural features and mRNA tissue distribution suggested that HGR213-1 might encode a dopamine D_1 receptor, membranes from transfected COS-7 cells were tested for their ability to bind to the D_1 selective antagonist [^{3}H]SCH23390. Untransfected COS-7 cells showed no specific binding of [^{3}H]SCH23390 (data not shown). Binding of [^{3}H]SCH23390 to

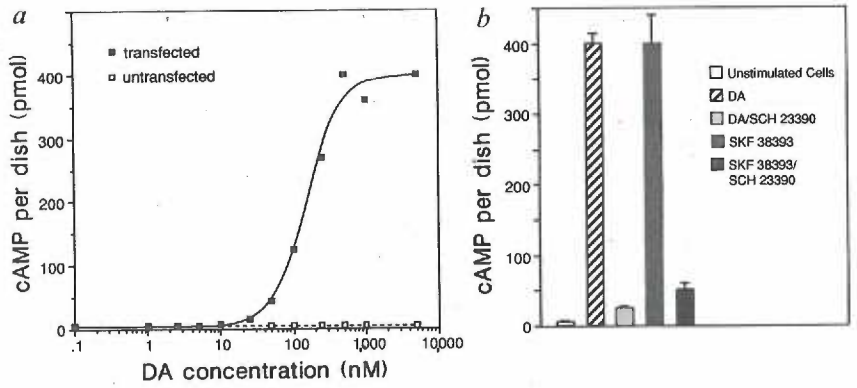


FIG. 4 *a*, Dopamine-induced cAMP accumulation in human embryonic kidney 293 cells transfected with HGR213-1. Intracellular cAMP was measured as a function of dopamine (DA) concentration after transient expression of HGR213-1. Triplicate plates were analysed for each point. The half-maximal stimulation concentration of dopamine (EC50) of the curve shown is 154 nM. *b*, Stimulation of cAMP accumulation in 293 cells by dopamine and SKF38393 and the antagonizing effect of SCH23390. Cyclic AMP production in 293 cells was stimulated by the agonists dopamine (125 nM) and SKF38393 (250 nM) and antagonized by SCH23390 (500 nM).

METHODS. Exponential growing human 293 cells (in 60-mm dishes) were transfected with HGR213-1 expression plasmid DNA (5 μg) in PBC12BI (ref. 27) using a modified calcium phosphate method[29]. The dishes were rinsed twice with DMEM plus 10% FCS after 18 h. Two days later, the plates were rinsed twice with DMEM containing 1 mg ml^{-1} BSA and 0.5 mM IBMX (3-isobutyl-1-methylxanthine). The cells were then incubated for 45 min at 37 °C in the same medium containing various drugs. After aspiration of the medium, cells were washed twice with ice-cold Hank's buffered saline and lysed with 1 ml of 60% ethanol. The cell debris was collected and pelleted and the supernatants lyophilized. The resulting pellets were resuspended in water and cAMP in each sample was quantitated using an assay method (Amersham) that measures the ability of cAMP in the sample to displace [8-^{3}H] cAMP from a high affinity cAMP binding protein[30]. The values obtained are normalized for the number of cells on a 60-mm dish (~5×10^5 cells in *a* and 10^6 cells in *b*).

membranes prepared from transfected COS-7 cells was saturable with a dissociation constant (K_d) of 0.3 nM (Fig. 3 inset). This value agrees well with both the reported value[28] and the value observed in parallel experiments with rat striatal membranes (data not shown). Figure 3 shows competition curves of various ligands with [^{3}H] SCH23390. The D_1 selective antagonist SCH23390 and agonist SKF82526 were most potent, and the D_2 selective antagonist haloperidol was virtually inactive. The rank order of ligand potency was: SCH23390 > (+)butaclamol > flupenthixol ≫ haloperidol. This pharmacological profile explicitly identifies the binding site as that of a dopamine D_1 receptor.

To demonstrate the HGR213-1 encodes a functional dopamine D_1 receptor we examined its ability to couple dopamine binding to activation of adenylyl cyclase. Human embryonic kidney 293 cells transiently expressing HGR213-1 were tested for their ability to respond to dopamine. When exposed to dopamine, untransfected cells showed no elevation of cAMP (Fig. 4*a*). In contrast, transfected cells displayed a concentration-dependent and saturable increase of intracellular cAMP levels with a half-maximal stimulation concentration (EC50) of about 125 nM (Fig. 4*a*). This value is comparable to the previously reported value[28]. SKF38393, a selective D_1 agonist, had a similar effect on the intracellular cAMP production and the stimulatory effects of both dopamine and SKF38393 were blocked by SCH23390 (Fig. 4*b*). These results indicated that the cloned D_1 receptor could couple positively to adenylyl cyclase.

On the basis of the above results we conclude that HGR213-1 encodes a human D_1 dopamine receptor. The successful cloning of the human D_1 dopamine receptor provides a new tool to study the regulation and function of this receptor. Moreover, the availability of both D_1 and D_2 dopamine receptor clones, which both bind to dopamine but couple to distinct effectors, should provide us with a new approach to address the complex interactions between these receptors. □

Received 26 April; accepted 15 June 1990.

1. Kebabian, J. W. & Caine, D. B. *Nature* **277,** 93–96 (1979).
2. Creese, I., Sibley, D. R., Hamblin, M. W. & Leff, S. E. *A. Rev. Neurosci.* **6,** 43–71 (1983).
3. Clark, D. & White, F. J. *Synapse* **1,** P347–P388 (1987).
4. Walters, J. R., Bergstrom, D. A., Carlson, J. H., Chase, T. N. & Braun, A. R. *Science* **236,** 719–722 (1987).
5. Waddington, J. L. *J. Psychopharmacol.* **3,** 54–63 (1989).
6. Lankford, K. L., DeMello, F. G. & Klein, W. L. *Proc. natn. Acad. Sci. U.S.A.* **85,** 4567–4571 (1988).
7. Bunzow, J. R. *et al. Nature* **336,** 783–787 (1988).
8. Saiki, R. K. *et al. Science* **239,** 487–491 (1988).
9. Dohlman, H. G., Caron, M. G. & Lefkowitz, R. J. *Biochemistry* **26,** 2657–2664 (1987).
10. Libert, F. *et al. Science* **244,** 569–572 (1989).
11. Boyson, S. J., McGonigle, P. & Molinoff, P. B. *J. Neurosci.* **6,** 3177–3188 (1986).
12. O'Dowd, B. F. *et al. J. biol. Chem.* **263,** 15985–15992 (1988).
13. Emorine, L. J. *et al. Science* **245,** 1118–1121 (1989).
14. Regan, J. W. *et al. Proc. natn. Acad. Sci. U.S.A.* **85,** 6301–6305 (1988).
15. Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S. & Dixon, R. A. F. *J. biol. Chem.* **264,** 13572–13578 (1989).
16. Lefkowitz, R. J. *et al. Cold Spring Harb. Symp. quant. Biol.* **53,** 507–514 (1988).
17. Jarvie, K. R., Booth, G., Brown, E. M. & Niznik, H. B. *Molec. Pharmacol.* **36,** 566–574 (1989).
18. Grandy, D. K. *et al. Proc. natn Acad. Sci. U.S.A.* **86,** 9762–9766 (1989).
19. Kobilka, B. K. *et al. Proc. natn Acad. Sci. U.S.A.* **84,** 46–50 (1987).
20. Frielle, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **84,** 7920–7924 (1987).
21. Cotecchia, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **85,** 7159–7163 (1988).
22. Kobilka, B. K. *et al. Science* **238,** 650–656 (1987).
23. Strader, C. D. *et al. J. biol. Chem.* **263,** 10267–10271 (1988).
24. Venter, J. C., Fraser, C. M., Kerlavage, A. R. & Buck, M. *Biochem. Pharmacol.* **38,** 1197–1208 (1989).
25. Kishimoto, A. *et al. J. biol. Chem.* **260,** 12492–12499 (1985).
26. Bouvier, M. *et al. Nature* **333,** 370–374 (1988).
27. Cullen, B. R. *Meth. Enzym.* **152,** 684–704 (1987).
28. Niznik, H. B. *et al. Molec. Pharmacol.* **34,** 29–36 (1990).
29. Chen, C. & Okayama, H. *Molec. cell. Biol.* **7,** 2745–2752 (1987).
30. Brown, B. L., Albano, J. D., Ekins, R. P. & Sgherzi, A. M. *Biochem. J.* **171,** 561–562 (1971).

ACKNOWLEDGEMENTS. We thank L. Robbins for help with cAMP measurements, K. Neve for suggestions, and C. Spear and L. Raymond for help with tissue culture. We thank R. Goodman and M. Forte for manuscript review and J. Tasnady and J. Shiigi for manuscript preparation. This work was supported by the NIH (O.C).