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Multiple human D_5 dopamine receptor genes: A functional receptor and two pseudogenes

(adenylyl cyclase/guanine nucleotide-binding protein-coupled receptor/gene amplification/termination codon/transient expression)

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ABSTRACT **Three genes closely related to the D_1 dopamine receptor were identified in the human genome. One of the genes lacks introns and encodes a functional human dopamine receptor, D_5, whose deduced amino acid sequence is 49% identical to that of the human D_1 receptor. Compared with the human D_1 dopamine receptor, the D_5 receptor displayed a higher affinity for dopamine and was able to stimulate a biphasic rather than a monophasic intracellular accumulation of cAMP. Neither of the other two genes was able to direct the synthesis of a receptor. Nucleotide sequence analysis revealed that these two genes are 98% identical to each other and 95% identical to the D_5 sequence. Relative to the D_5 sequence, both contain insertions and deletions that result in several in-frame termination codons. Premature termination of translation is the most likely explanation for the failure of these genes to produce receptors in COS-7 and 293 cells even though their messages are transcribed. We conclude that the two are pseudogenes. Blot hybridization experiments performed on rat genomic DNA suggest that there is one D_5 gene in this species and that the pseudogenes may be the result of a relatively recent evolutionary event.**

The dopamine receptors are integral membrane proteins that interact with guanine nucleotide-binding proteins (G proteins) to transduce dopamine stimulation into intracellular responses. The majority of dopamine receptors are concentrated in the mesocortico–mesolimbic, nigrostriatal, and tuberoinfundibular pathways. These neuronal circuits are known to influence mood, behavior, initiation of movement, and prolactin secretion. Prior to the cloning of the rat D_2 dopamine receptor gene (1) only D_1 and D_2 receptors were generally acknowledged to account for the diverse physiological effects of dopamine (2–4), although some pharmacological evidence was inconsistent with this view (5).

With nucleic acid probes based on the sequence of the cloned rat D_2 receptor gene it was quickly revealed that at least four dopamine receptor genes exist in humans and rats (6–15). Based on their genomic organization these genes can be divided into two types: those with (D_2, D_3, and D_4) and those without (D_1) introns in their coding regions. During a structural analysis of the human D_1 dopamine receptor gene we obtained evidence that the human genome contains a sequence that is similar but not identical to the D_1 gene. To determine whether or not this sequence encoded a dopamine receptor, we undertook its cloning and characterization.**

MATERIALS AND METHODS

Genomic Library Screening, DNA Sequencing, and DNA and RNA Blot Hybridization Analysis. High molecular weight human genomic DNA was prepared from leukocytes (16) and 5 μg was digested with *Eco*RI and electrophoresed in a 0.7% agarose gel. The 5- to 6-kilobase (kb) region was excised and the DNA was purified using GeneClean (Bio 101, La Jolla, CA), ligated to λgt10 arms (Stratagene), and packaged *in vitro* using Gigapack packaging extracts (Stratagene). The library was replicated on duplicate nylon filters (DuPont/NEN) and probed with a nick-translated 3-kb genomic *Eco*RI–*Sac* I fragment containing the coding region of the human D_1 dopamine receptor (9). The reported nucleic acid sequences were determined in both orientations (Sequenase, United States Biochemical). Sequence analysis was aided by Intelligenetics and Genetics Computer Group software. RNA was prepared by the guanidinium isothiocyanate method and analyzed as described (1). High molecular weight human and rat genomic DNA was Southern blotted to nitrocellulose (Schleicher & Schuell) as described (17).

Polymerase Chain Reaction (PCR) and Expression Cloning. Twenty-five cycles of PCR (strand separation for 1 min at 95°C, annealing for 2 min at 55°C, and extension for 3 min at 72°C) were performed in a Coy Tempcycler using 1 unit of Replinase (NEN), 1 ng of template DNA, and 50 pmol of each synthetic oligodeoxynucleotide primer (5′-CCGAATTCGCCTTCGACATCATGTGC-3′ and 5′-CCGGATCCGTCACGATCATGATGGC-3′). The PCR product was gel-purified, digested, ligated into M13 phage, and sequenced. For expression studies, DNAs were synthesized by PCR from HGRI-4, -6, and -8 templates with the primers 5′-CCGTCGACGATCGCGCACAAACCGAC-3′ and 5′-CCGTCGACAGTACTGGAAAGGCATGTAT-3′. The 1.56-kb products were cloned into the transient expression vector pBC12BI (18).

Expression and Pharmacological Evaluation. A modified calcium phosphate method (19) was used for the transfection of COS-7 and 293 cells. Forty-eight hours after transfection, COS-7 cells were harvested as described (9). Binding assays were performed in duplicate and competition curves in triplicate. Reaction mixtures contained 50 mM Tris buffer (pH 7.4), 0.9% NaCl, 0.025% ascorbic acid, 0.001% bovine serum albumin, and various concentrations of [^{3}H]SCH23390 (Amersham, 69 Ci/mmol; 1 Ci = 37 GBq). Membranes (5–50 μg) and [^{3}H]SCH23390 (0.7–1.0 nM) were combined with various concentrations of unlabeled drugs at 37°C for 1 hr. Nonspecific binding was defined in the presence of 5 μM (+)-butaclamol. Samples were filtered through glass-fiber filters (Schleicher & Schuell, no. 32) and the retained material was washed three times with 4 ml of ice-cold 50 mM Tris buffer (pH 7.4) prior to liquid scintillation counting (Packard

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Abbreviations: G protein, guanine nucleotide-binding protein; G_s, stimulatory G protein; TMD, transmembrane domain.

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**The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M67439–M67441).

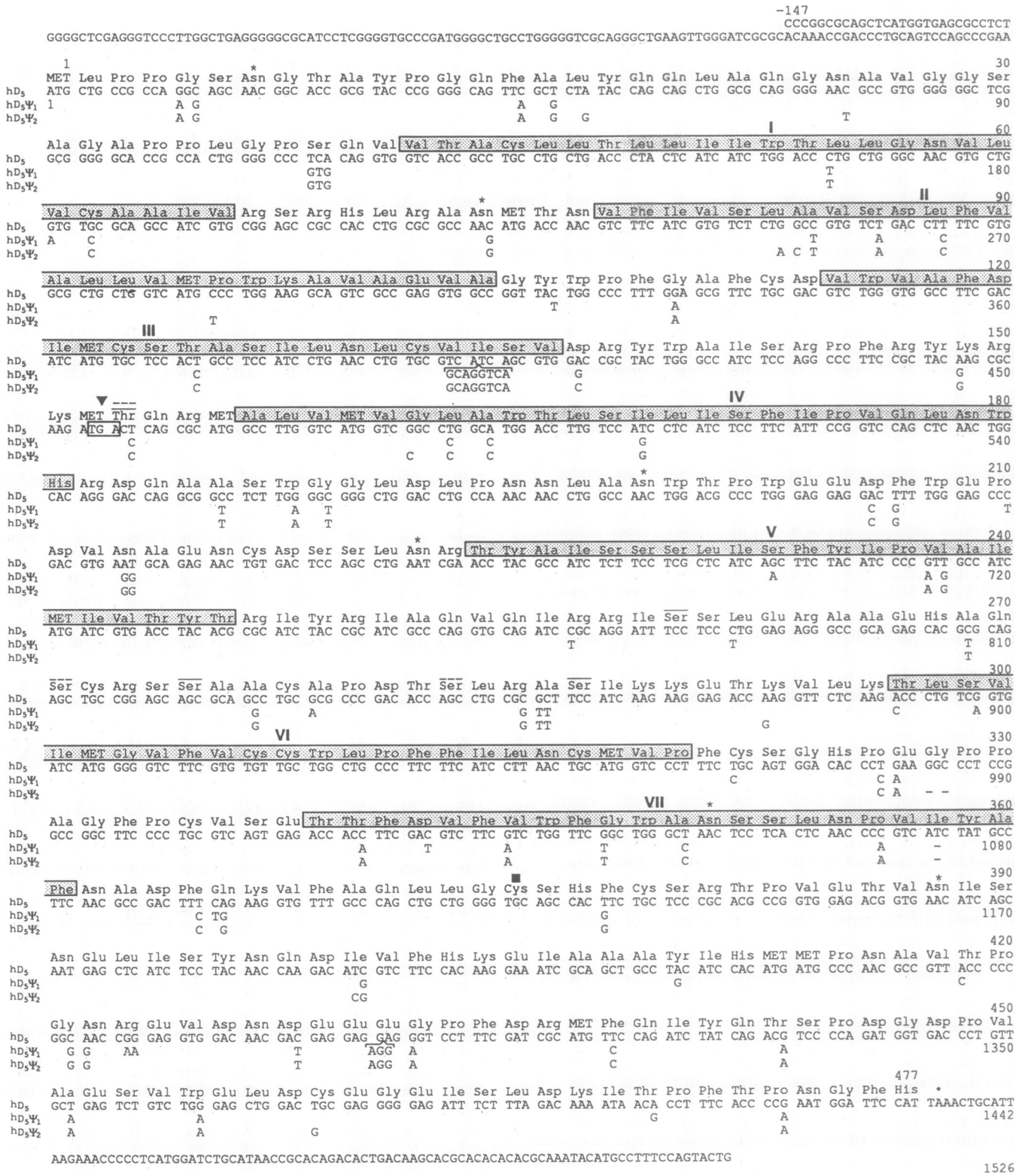

FIG. 1. Nucleotide and deduced amino acid sequences of the human D_5 receptor gene, hD_5 (HGRI-4), and the two pseudogenes, $hD_5\psi_1$ (HGRI-6) and $hD_5\psi_2$ (HGRI-8). Only the nucleotide differences in the coding region are given for the pseudogenes, with dashes indicating deletions and expanded carets, insertions. The in-frame stop codon is boxed with a solid triangle over it. The seven proposed transmembrane domains (TMDs) are shaded and numbered I–VII. Putative N-glycosylation sites are indicated by stars, protein kinase A phosphorylation sites are overlined, and protein kinase C sites are overlined with a dashed line. A solid box identifies the cysteine residue that may be palmitoylated. The D_5 stop codon is identified by a dot.

2200CA TriCarb). The GRAPHPAD computer program was used for data analysis and curve fitting with K_i values determined as described (9). The cAMP assays were performed on 293 cells as described (9).

RESULTS

Cloning and Sequencing of the Human Genes. The human D_1 receptor gene (9) was used to probe a Southern blot of

human genomic DNA. Autoradiography revealed that it hybridized to a 5-kb *Eco*RI fragment and was washed off at higher temperatures. A human genomic library enriched in 5- to 6-kb *Eco*RI fragments was prepared in λgt10 and screened with the human D_1 receptor gene. Of the many positive plaques, DNAs from 10 were prepared and each was found to contain a 5-kb insert. One of these clones, HGRI-6, was further characterized by sequencing (Fig. 1). At the nucleotide sequence level HGRI-6 is 65% identical to the human D_1 receptor gene. The nucleotide sequences encoding the seven putative TMDs are 74% identical to those of the human D_1 receptor (9). However, HGRI-6 contains an in-frame stop codon, suggesting that it is a receptor pseudogene ($hD_5\psi_1$).

To establish whether the human genome contains a functional homologue to HGRI-6, we performed PCR on DNA prepared from nine more genomic clones. Nucleotide sequence analysis of the PCR products revealed two additional unique genomic clones, HGRI-4 and HGRI-8. The nucleotide sequences of their putative coding regions are 95% and 98% identical to that of HGRI-6, respectively (Fig. 1). Translation of the HGRI-8 sequence revealed that it also contains an in-frame stop codon ($hD_5\psi_2$). Of the three genes, only HGRI-4 (hD_5) has an open reading frame that is sufficiently long to encode a G-protein-coupled receptor (Fig. 1). The deduced protein consists of 477 residues (relative molecular mass, M_r = 52,950) with seven putative TMDs. Six asparagines are potential N-linked glycosylation sites and the presence of one of them near the amino terminus of the protein (Asn^6) is a structural feature shared by all of the cloned dopamine receptors. Another potential N-glycosylation site, Asn^{351}, is unusual in that it is located in a TMD. Interestingly, both human and rat D_1 receptors also display this feature. The two cysteine residues Cys^{113} and Cys^{217} are conserved among dopamine receptors and may be important for stabilizing the receptor's tertiary structure. Several potential protein kinase phosphorylation target sites are present in the deduced receptor sequence: four for protein kinase A (20) and three for protein kinase C (21). Five of these are located in the putative third cytoplasmic loop of the molecule. This domain is thought to be important for the coupling of the receptor to G proteins (22), which suggests that these sites may serve a regulatory function. In addition, there are several serines and threonines in the carboxyl-terminal portion of the protein that may be phosphorylated by receptor kinase (23). Also, Cys^{375} may be palmitoylated, providing the receptor with a point of attachment to the plasma membrane (24, 25).

Comparison of the HGRI-4 deduced amino acid sequence with the four other published human dopamine receptors reveals significant conservation in their putative TMDs. Overall the seven TMDs of HGRI-4 are 82% identical to the human D_1 dopamine receptor. Noteworthy among the conserved residues are Asp^{120}, Ser^{229}, and Ser^{233}, which are found in TMDs III and V of all the cloned catecholamine receptors. These residues are thought to coordinate the amino and catechol hydroxyl moieties of catecholamine ligands (26, 27). In addition, the putative third cytoplasmic loop and carboxyl tail of HGRI-4 are similar in size to their counterparts in the human D_1 dopamine receptor. Taken together, these structural characteristics strongly suggest that HGRI-4 could encode a D_1-like dopamine receptor that may couple to the stimulatory G protein (G_s).

Expression and Pharmacological Evaluation. To establish the pharmacological profile of this putative receptor, the gene was cloned into the vector pBC12BI and transiently expressed in COS-7 monkey kidney cells, which lack [^{3}H]SCH23390 binding sites (9). The putative pseudogenes, HGRI-6 and HGRI-8, as well as the human D_1 dopamine receptor gene were also cloned into pBC12BI. Membranes prepared from cells transfected with each of the two putative pseudogenes failed to bind [^{3}H]SCH23390 (data not shown) even though the HGRI-6 and -8 mRNA was expressed at levels equivalent to the levels of pBCHGRI-4 (Fig. 2). In contrast, membranes prepared from cells transfected with pBCHGRI-4 expressed high levels of saturable [^{3}H]SCH-23390 binding upon Scatchard analysis, with an average dissociation constant, K_d, of 0.35 nM (n = 7) and an average B_{max} of 4 pmol per mg of protein (n = 7) (Fig. 3*A Inset*). These values are similar to those obtained in parallel experiments with the human D_1 dopamine receptor.

To pharmacologically characterize this D_1-like binding site, experiments were performed with agonists and antagonists in competition with [^{3}H]SCH23390 (Fig. 3*A*). The antagonist data were fit best by assuming one class of binding site. Based on these studies the rank order of antagonist potency (K_i) was SCH23390 (0.6 nM) > (+)-butaclamol (9.1 nM) > *cis*-flupenthixol (23.6 nM) > haloperidol (156 nM) > clozapine (406 nM) >> (−)-butaclamol (>10 μM). This is the same relative rank order of potency as previously observed for the human D_1 receptor (9). When the agonists were evaluated all three competition curves (Fig. 3*A*) had slopes that were <1 (0.6). Based on these results the data were fit by assuming the presence of both high- and low-affinity sites. The rank order of potency for the agonists was SKF82526 (fenoldopam) > SKF38393 > dopamine, with average (n = 3) K_i values of 0.6 nM and 27 nM, 0.52 nM and 469 nM, and 12.8 nM and 1806 nM, respectively. In parallel experiments the human D_1 receptor also displayed high and low affinity for dopamine, but the average (n = 2) K_i values, 32 nM for the high-affinity site and 9109 nM for the low-affinity site, were higher than those observed for the D_5 dopamine receptor. These pharmacological data strongly suggest that pBCHGRI-4 encodes a D_1-like binding site, D_5, with a high affinity for dopamine.

Whether or not D_5 is a functional receptor was examined by its ability to stimulate intracellular cAMP accumulation in the human embryonic kidney cell line 293. Cells expressing pBCHGRI-4 displayed a concentration-dependent and saturable increase in intracellular cAMP levels when exposed to dopamine (Fig. 3*B*). Furthermore, this stimulation could be antagonized by 250 nM SCH23390 (Fig. 3*B Inset*). Of particular interest is the shape of the dopamine/cAMP dose–response curve. The data from three independent experiments were best fit by a two-site model, and two half-maximal stimulation concentrations (EC_{50}) for dopamine were calculated: 5.0 nM and 275 nM. In parallel experiments,

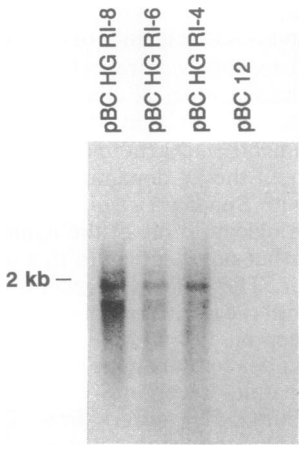

FIG. 2. Northern blot analysis of the human D_5 genes transiently expressed in COS-7 cells. Total RNA was prepared from cells transfected with pBCHGRI-8, pBCHGRI-6, pBCHGRI-4, or pBC12BI (vector control). Each lane contained 5 μg of total RNA. Hybridization was with a ^{32}P-labeled fragment spanning TMDs III–V of HGRI-4.

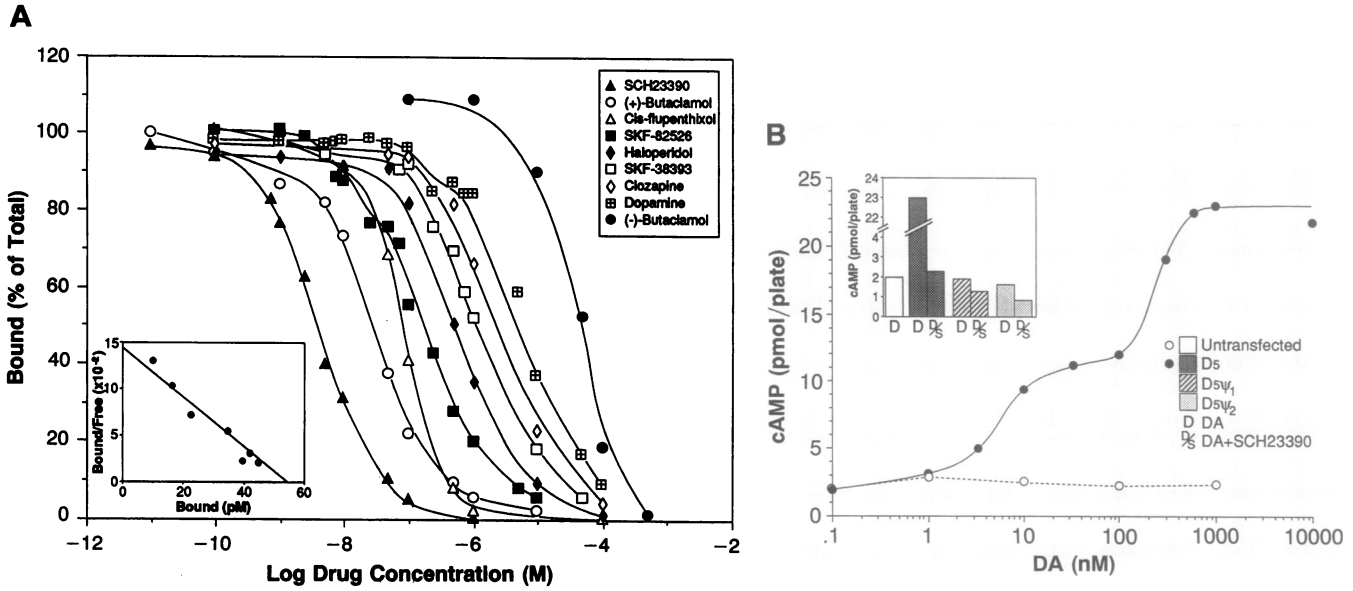

FIG. 3. Pharmacological profile and second-messenger coupling of the human D_5 genes. (*A*) Binding of [^{3}H]SCH23390 to membranes prepared from COS-7 cells transiently expressing pBCHGRI-4. (*Inset*) Scatchard transformation of the saturation binding data. (*B*) Dopamine (DA)-stimulated cAMP accumulation in human 293 cells. A representative dose–response curve is shown with each point the average of duplicate plates. (*Inset*) SCH23390 (250 nM) was used to antagonize both dopamine-stimulated and SKF38393-stimulated cAMP production in 293 cells. Values are the average of two independent experiments.

293 cells transfected with the two putative pseudogenes pBCHGRI-6 and -8 showed no significant accumulation of intracellular cAMP when exposed to agonist (Fig. 3 *Inset*).

Genomic Analysis of Human and Rat D_5 Genes. To investigate whether multiple D_5 genes are unique to humans we performed a Southern blot analysis of both rat and human genomic DNA. With the human D_5 probe, very strong signals were seen in the human lanes with weaker signals seen in the rat lanes (Fig. 4*A*). To demonstrate that the signal intensities corresponded to gene copy number and not species differences between the rat and human genes, the filter was stripped of human probe and exposed to a rat D_5 gene probe (D.K.G., unpublished data). The signals obtained with the two probes were essentially equivalent (Fig. 4*B*).

DISCUSSION

In this report we present the cloning and expression of a functional human dopamine receptor, D_5, and two related pseudogenes, $D_5\psi_1$ and $D_5\psi_2$. While this manuscript was in preparation the amino acid sequence of a human D_5 dopamine receptor identical to ours was reported (28). However, in addition to the pharmacological characterization of the D_5 dopamine receptor we present the results of second-messenger experiments and genomic analyses that expand our knowledge about the D_5 dopamine receptor system.

In agreement with Sunahara *et al.* (28) our data indicate that the pharmacological profile of the human D_5 dopamine receptor is similar but not identical to that of the human D_1 dopamine receptor. The human D_5 receptor binds agonists and antagonists with the same rank order of potency as does the human D_1 receptor, but its high- and low-affinity sites display higher affinities for dopamine than the corresponding sites in the D_1 dopamine receptor.

Furthermore, human D_5 differs from D_1 in its dose-dependent stimulation of adenylyl cyclase. D_5 receptors are able to stimulate adenylyl cyclase in 293 cells, but unlike the D_1 receptor (9), the data from these dose–response experiments are fit best to a biphasic curve with two EC_{50} values for dopamine of 5 nM and 275 nM. Although the physiological significance of this biphasic dose response is difficult to assess without further investigation, it is tempting to relate the two EC_{50} values to the high- and low-affinity states of the D_5 receptor as seen in the binding experiments. However, if a portion of the dose–response curve were due to the low-affinity form of the receptor, it would be difficult to reconcile with the widely held view that only the high-affinity form of the receptor couples to G_s. Another possibility is that in 293 cells the D_5 dopamine receptor may stimulate adenylyl cyclase by coupling, with different affinities, to more than one type of G-protein α subunit. Such an effect could be mediated by the receptor's third cytoplasmic loop, a domain thought to be involved in coupling the receptor to the G protein complex (22). One consequence of this model is that the receptor may regulate several different effector enzymes. Therefore D_5 may not only stimulate adenylyl cyclase directly through G_s but also indirectly through G_p, which couples to phospholipase C, as is the case for the muscarinic acetylcholine receptor subtypes M_1 and M_3 (29). Finally, it is possible that we are observing a novel response of the adenylyl cyclase

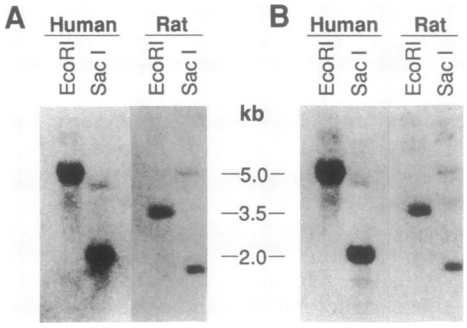

FIG. 4. Southern blot analysis of human and rat D_5 receptor genes. Each lane contained 3 μg of human or rat genomic DNA digested with *Eco*RI or *Sac* I restriction endonuclease. (*A*) Autoradiogram of the filter after it was probed with a fragment from HGRI-4 spanning TMDs III–V. (*B*) Autoradiogram of the same filter after it was stripped (15 mM NaCl/1.5 mM trisodium citrate, pH 7/0.1% SDS, 80°C) and probed with a rat D_5 fragment spanning TMDs III–V (D.K.G., unpublished data). The faint signal visible with both D_5 probes in the human and rat DNA cut with *Sac* I is due to cross-hybridization with the D_1 receptor gene.

present in 293 cells. The recent cloning and expression of several different forms of adenylyl cyclase has revealed that the activity of these enzymes is regulated in a complex manner. Indeed, the activity of the type I (calmodulin-sensitive) form of the enzyme has been shown to be stimulated by α_s subunits and inhibited by $\beta\gamma$ subunits (30). In contrast to the type I form of the enzyme, which is predominantly expressed in brain tissue, the type II enzyme is ubiquitously expressed (including in 293 cells) and may respond differently to $\beta\gamma$ (30). If, for example, the type II enzyme were stimulated by $\beta\gamma$, the resultant effect on cyclase might be to potentiate the effects of α_s. If this model is correct, then all receptors that couple to G_s in 293 cells should display a biphasic dose–response curve. We failed to observe this phenomenon with D_1 (9); however, its stimulation of cAMP accumulation was so robust (200-fold) that a subtle response may have been overlooked. With this in mind it will be of interest to reexamine the dose response of D_1 in more detail.

The cloning of multiple human D_5 genes raises many questions, including how many D_5 genes there are in humans and how and when they arose during evolution. *In situ* hybridization to human metaphase chromosomes has revealed that three distinct loci, located on chromosomes 1 (1q21), 2 (2q11.2), and 4 (4p16), hybridize to the human D_5 probe (D.K.G. and L.A., unpublished data). Our data indicate that we have cloned three genes that represent at least two of these three loci. There is still the possibility that the two pseudogenes are allelic, leaving another sequence (representing the third locus) yet to be identified. How these genes came to be duplicated is unknown, but the possibility of either transposition or viral retroposition (31) exists. Finally, our interpretation of the Southern blotting data, that rats possess only one copy of a D_5-like gene per haploid genome, suggests that the duplication event(s) may have occurred at some time after the divergence of the primates.

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