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Differential expression of the mouse D_2 dopamine receptor isoforms

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We have identified and characterized the cDNAs corresponding to the mouse D_2 dopamine receptors. We show that in the mouse the D_2 dopamine receptor is found in two forms, generated by alternative splicing of the same gene. mRNA distribution analysis of areas expressing the D_2 receptors shows that the larger form is the most abundant, except in the brain stem where the shorter form is predominant. Membranes of mammalian cells transiently transfected with both forms of D_2 receptor bind [^{3}H]spiperone with a high affinity.

Mouse D_2 dopamine receptor; mRNA distribution; Expression

1. INTRODUCTION

Dopamine receptors can be divided into two classes D_1 and D_2 [1,2]. D_1 receptors activate the adenylyl cyclase pathway while D_2 receptors act as inhibitors. The dopamine D_2 receptor is highly represented in the central nervous system, especially in areas like the striatum and substantia nigra as well as in the pituitary gland [3,4]. Abnormal regulation of the receptor's levels seems to be associated with disorders such as Parkinson's disease [5] and schizophrenia in humans [6]. In the anterior pituitary gland dopamine acts through a D_2 type receptor and causes inhibition of prolactin production and release [7]. Activation of the D_2 receptor lowers the intracellular level of cAMP by a mechanism which appears to involve coupling of the receptor to an inhibitory G protein (G_i) [8]. The gene for a D_2 dopamine receptor has been cloned from rat brain; it encodes a predicted protein of 415 amino acids (aa) [9] which belongs to the family of G protein-coupled membrane receptors [10]. Recently a longer cDNA isoform encoding a predicted protein of 444 aa has been cloned from rat, human and bovine tissues [11–16].

We have isolated a cDNA from a mouse pituitary library encoding the 444 aa form of the D_2 receptor, here referred to as D_2A. This cDNA is highly homologous at the nucleotide and amino acid level to both forms of the rat D_2 receptor. Analysis of mouse genomic DNA suggests that the two forms originate by alternative splicing. We have analyzed the distribution of the two forms of D_2 dopamine receptors in the mouse brain. The results show that the shorter form, here referred to as D_2B, is the least abundant in most CNS areas, with the exception of the brain stem where it is predominant.

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2. MATERIALS AND METHODS

A mouse pituitary cDNA library was constructed in λzapII (stratagene) starting from 3 μg polyA$^+$ RNA from female mice pituitaries. The cDNAs were synthesized with a cDNA cloning kit from Pharmacia, containing *EcoRI-NotI* adaptors at each site, inserted into the *EcoRI* site of λzapII arms. The library (1 10^6 pfu) was transferred on Nitrocellulose filters (Schleicher and Schuell) and screened with two degenerate ^{32}P-labelled oligonucleotides (Spec. act. 10^8/μg) specific to conserved sequences present in the

VI (5'- TT$^{CACCC}_{TGTGG}$ATCTGCTGGCTGCCCTTCTTC 3')

and VII

(5'-TGGCT$^{T\ G}_{A\ T}$GGCTA$^{T\ T}_{C\ C}$CAA$^{TAG}_{CTC}$ 3')

transmembrane domains of receptors binding cationic amines [17]. The hybridization conditions were 5×SSC, 5× Denhardt's, 0.1% sodium pyrophosphate, 1% SDS and salmon sperm DNA (100 μg ml^{-1}) at 42°C. Filters were washed in 2×SSC and 0.1% SDS at 42°C. 3 overlapping clones were rescued from the λ zap vector and sequenced on both strands by the Sanger dideoxy chain termination method using Sequenase (US Biochemical Corporation) [18]. To obtain the cDNA corresponding to the shorter form of the mouse D2 Dopamine receptor, D_2B, cDNA was synthesized from 1 μg of total RNA from mouse brain stem in a 20 μl reaction containing 50 mM Tris-HCl, pH 8.3, 150 mM KCl, 10 mM $MgCl_2$, 15 mM DTT, 0.5 mM dNTP, 0.5 μg of amplimer A (5'-GGCCTTTCTGCCCACAG-CTT-3') complementary to the D_2A sequence from nucleotide 1404 to 1423 of the coding sequence and 10 U AMV reverse transcriptase. After incubating at 42°C for 1 h, reactions were inactivated by heating at 65°C for 10 min and 4 μl were added to a PCR reaction.

The PCR reactions were made in 50 μl containing 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTP, 0.5 μg amplimer A and 0.5 μg amplimer B (5'-GTCACCTGCTCI-GTCTAT-3') complementary to the D_2A sequence from nucleotide 609 to 628 of the coding sequence. Reactions were heated at 94°C for 2 min, then 2.5 U of Taq polymerase were added and the temperature was cycled with the following profile: 94°C, 0.5 min; 55°C, 1 min, 72°C, 2 min. After 40 cycles the PCR reaction was digested with *Hinc* II that liberates a 498 bp long fragment. A *Hinc* II digestion was also performed on the D_2A cDNA. We exchanged the 498 bp fragment specific to the D_2B form with the 585 bp long fragment of the D_2A form. The cDNA obtained was sequenced to ensure the absence of PCR artefacts.

Quantitative S_1 nuclease analysis was performed on 10 μg of total RNA, prepared by the LiCl-urea method [19]. The hybridization conditions were 50% formamide, 40 mM Pipes (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl at 37°C for 12 h. Digestions were made in 0.28 M NaCl 0.05 M sodium acetate (pH 4.6), 4.5 mM $ZnSO_4$, with 300 U S_1 (Appligene, Strasbourg, France) at 25°C for 2 h. The digestions were located on a 8% polyacrylamide/urea gel.

For eukaryotic expression, cDNA fragments containing the entire coding region for the mouse D_2A and D_2B receptor were inserted in both orientation in the pSG5 vector [20]; to created $pSVD_2A^+$ (sense orientation) or $pSVD_2A^-$ (antisense orientation) and $pSVD_2B^+$ or $pSVD_2B^-$. Cos7 cells were transfected with either $pSVD_2A^+$ or $pSVD_2B^+$ (10 μg DNA/plate) by the calcium phosphate method [21], washed after 12 h and harvested after 36 h. Membranes were prepared as already described [22]. Binding assays were carried out with 40 μg protein/sample [22] using [^{3}H]spiperone and bromocriptine at the concentration indicated in Fig. 4. Data were analysed by non-linear least squares regression [23].

3. RESULTS

3.1. *Cloning of the D_2 dopamine receptor cDNAs*

Two sets of oligonucleotides were generated to screen a mouse pituitary cDNA library. The sequence of the oligonucleotides was based on a comparison of the

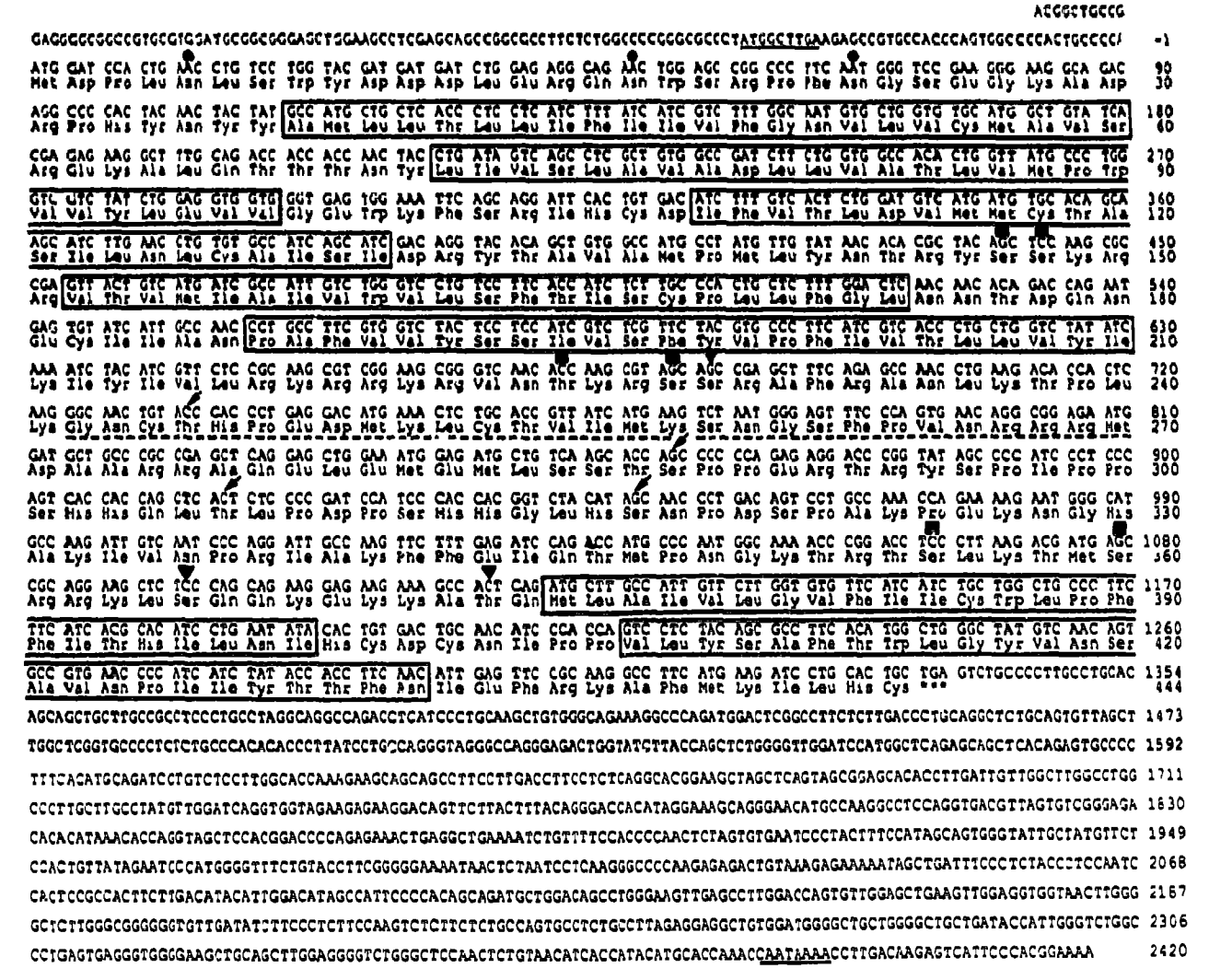

Fig. 1. Sequence analysis of the mouse Dopamine D_2A receptor cDNA. Nucleotide and predicted amino acid sequences are shown and numbered in the right hand margin beginning with the first methionine of the open reading frame. Asterisks (*) indicate potential N-linked glycosylation sites. Putative protein kinase A sites are indicated (□). Triangles (△) indicate putative protein kinase C sites, casein kinase II sites are designated by an arrow. The additional 29 aa specific to this isoform are underlined with dashed lines. The poly-A adenylation site is underlined as well as a small open reading frame in the 5' untranslated region in frame with the first ATG. The putative 7 transmembrane domains are boxed.

amino acid sequences of G protein-coupled receptors binding cationic amines (for review see ref. 17). A cDNA was isolated of 2549 bp with an open reading frame coding for a 444 aa long protein (Fig. 1). The sequence was recognized as encoding a D_2 receptor because of its high homology to the D_2 receptor isolated from rat brain cDNA [9], except for an insertion of 87 base pairs in the region corresponding to the putative third intracytoplasmic domain. A similar form of the D_2 receptor has been characterized in other species [11-16].

Southern genomic blot analysis using specific oligonucleotides showed the presence of a single band suggesting that the two cDNAs are products of the same gene (Fig. 2), and they are likely to be generated by alternative splicing as it has been shown in rat and humans [12,13,15].

3.2. *mRNA distribution analysis*

In order to gain insights into the function of the two D_2 dopamine receptor isoforms, we investigated their relative distribution in areas in which the D_2 receptor is expressed. Therefore we performed a quantitative S1 nuclease mapping on mRNAs from pituitary and different areas of the brain of adult mice (Fig 3). A 75-mer oligonucleotide was synthesized spanning the region of the putative third intracytoplasmic domain, where the insertion is located. Experiments were performed in probe excess, to ensure a correct quantification of the data. This analysis shows that in pituitary and striatum D_2A is at least 3-fold more abundant than D_2B. Interestingly this ratio is completely inversed in the brain stem where D_2B is predominant (see Fig 3; D_2A and D_2B). The differential distribution of the D_2 mRNAs in the brainstem obtained by S_1 nuclease analysis, reveals a difference in the ratio D_2A:D_2B which has not been detected before by Northern or PCR analysis. Northern analysis is not suitable for a direct quantification of the relative presence of the two forms of D_2 receptors, because of the lack of sequences specific to the D_2B isoform. PCR analysis [13] also failed to reveal such a difference, probably due to a lack of quantification in the PCR reactions, in which it is likely that the longer form is better amplified, as it always appears predominant.

3.3. *Functional expression*

To determine whether D_2A and D_2B cDNAs encoded functional receptors we introduced the sense or antisense expression vectors by transient transfection in the Cos7 cells. Membranes from transfected and non-transfected cells were tested for their ability to bind [^{3}H]spiperone [22], a D_2 specific ligand. Binding of [^{3}H]spiperone to these membranes was reversible saturable and with high affinity for both types of receptors (data not shown). Fig. 4 demonstrates the ability of a dopaminergic ligand, bromocriptine, to compete with high affinity for specific [^{3}H]spiperone binding to transfected Cos7 cell membranes (D_2A $IC_{50} = 5 \cdot 10^{-8}$ M; D_2B $IC_{50} = 8 \cdot 10^{-8}$M). Untransfected Cos 7 cells as well as those transfected with the antisense constructs were unable to bind [^{3}H]spiperone, ruling out the possibility that an endogenous dopamine receptor could be responsible for the observed binding.

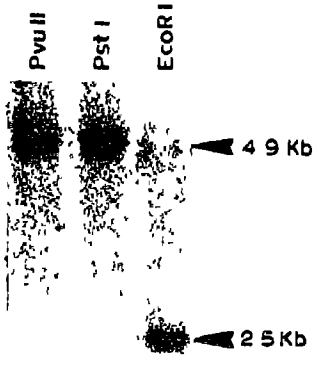

Fig 2 Southern blot analysis of mouse genomic DNA 10 μg of mouse genomic DNA were digested by either *PstI*, *PvuII* or *EcoR*I as indicated on each lane After transfer the filter was hybridized with ^{32}P-labelled oligonucleotides complementary to either D_2A (from nucleotide 724-784) or D_2A and D_2B (from nucleotide 843-903) The approximative size of the single bands detected by hybridization with either probes, is as indicated

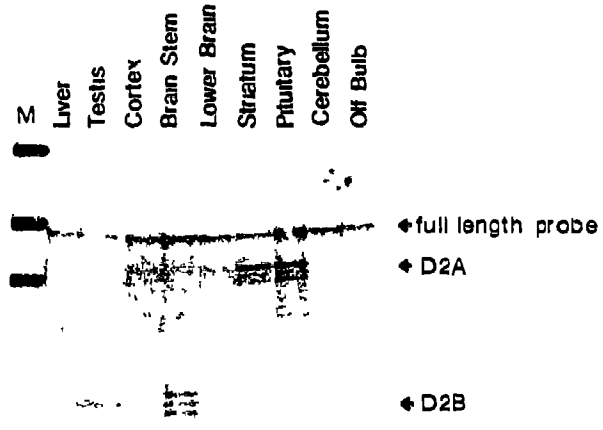

Fig 3 Quantitative S1 nuclease analysis of mouse brain and pituitary mRNA 10 μg of total mRNA were hybridized to a synthetic 75-mer oligonucleotide, ^{32}P-end labelled by T4 polynucleotide kinase, complementary to the D_2 receptors mRNA from nucleotide 792 to 860 to which a 6 nucleotides non-specific tail was added (spec act 10^8/μg). This probe was homologous for 51 nucleotides to both forms (415-444) of D_2 receptors while 18 nucleotides were specific to the D_2A (444) form The protected fragments were 51 nucleotides long ($D_2$415) and 69 nucleotides long ($D_2$444) as expected, the 75 nucleotides full-length probe is indicated The marker used is a ^{32}P-labelled pBR 322 digested by *MspI*, the size of the fragments shown in the picture are in order 90, 76 and 67 base pairs The tissue from where the mRNA was extracted are indicated above each lane The relative amount of mRNA corresponding to either form of D_2 receptor was estimated by densitometric scanning of the autoradiogram

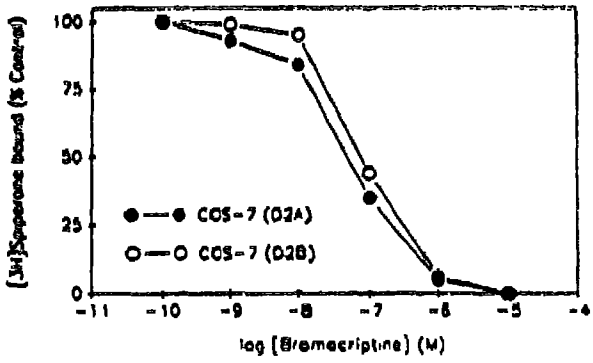

Fig. 4. Binding assay. Competition analysis of the dopaminergic ligand bromocriptine for [^{3}H]spiperone (0.5 nM) binding in cos 7 cells. Bromocriptine concentrations are as indicated in the figure.

4. DISCUSSION

The D_2 dopamine receptor has been shown to generate the activation of different signal transduction pathways upon binding to its ligand [24]. These effects are mediated through its interactions with at least two G proteins [25]. In this paper we report the isolation of two forms of the D_2 dopamine receptors from mouse, D_2A and D_2B. These two forms generated by alternative splicing differ by an insertion of 29 aa between position 241 and 271 in the putative third intracytoplasmic domain of the D_2B receptor. This domain has been shown to play an important role in the coupling to G proteins in the case of the β_2- and α_2-adrenergic receptors [26]. The finding of two isoforms for the dopamine D_2 receptor, that differ only in this domain, raises the possibility of a differential coupling of these two receptors to G proteins. The two dopamine D_2 receptor amino acid sequences are highly conserved, including the 29 residue insertion, 100% from mouse to rat (97% homology at the nucleotide level, in the translated region) and 95.7% from rodents to human (90.45% at the nucleotide level, in the translated region), suggesting functional constraints on these sequences.

In order to gain insight into their function, we analyzed the RNA distribution of the D_2 receptor's isoforms. The D_2A form is the most abundant throughout all the areas tested with the exception of the brainstem, where the D_2B form is predominant. This finding points out the interesting possibility that the D_2B form could represent the presynaptic D_2 auto-receptor, able to regulate dopamine synthesis and release, since the brainstem contains the nuclei (substantia nigra) from where the dopaminergic fibers arise. We could not detect the presence of D_2 receptors in the adult mouse liver, like others did in rat [13]. We do not know whether this is due to the limit of detection of our probe, or whether the other authors used a fetal liver, as it was not specified in their paper. Concerning their binding affinity, classical pharmacological studies did not permit to discriminate between them as both receptors seem to have the same affinity for the ligand tested, when transfected in cos7 cells, as well as in other cell lines [12,13,14].

It has to be taken into account that the cell lines used could be deficient or limiting in one of the specific G proteins that bind to these receptors [27]. Alternatively, the high concentration of DNA transfected into these cells, necessary to observe binding, could result in high levels of receptors that would out number the available G proteins; thus the binding observed would correspond mostly to receptors that are not coupled to G proteins. Alternatively the difference between these two receptors could reside in the activation of several second messenger pathways in a differential manner. For instance, it has been shown that the α_2-adrenergic receptors can both activate phospholipase C and inhibit adenylate cyclase [28].

Further studies will be aimed to detect a functional difference between the two forms of the dopamine D_2 receptors: measuring the effect of their expression on the adenylyl cyclase as well as on other signal transduction pathways in different cell lines.

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