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The dopamine D_2 receptor: two molecular forms generated by alternative splicing

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Cloned human dopamine D_2 receptor cDNA was isolated from a pituitary cDNA library and found to encode an additional 29 amino acid residues in the predicted intracellular domain between transmembrane regions 5 and 6 relative to a previously described rat brain D_2 receptor. Results from polymerase chain reactions as well as *in situ* hybridization revealed that mRNA encoding both receptor forms is present in pituitary and brain of both rat and man. The larger form was predominant in these tissues and, as shown in the rat, expressed by dopaminergic and dopaminoceptive neurons. Analysis of the human gene showed that the additional peptide sequence is encoded by a separate exon. Hence, the two receptor forms are generated by differential splicing possibly to permit coupling to different G proteins. Both receptors expressed in cultured mammalian cells bind [^{3}H]spiperone with high affinity and inhibit adenylyl cyclase, as expected of the D_2 receptor subtype.

Key words: adenylyl cyclase/alternative splicing/dopamine D_2 receptor/molecular cloning/receptor subtypes

Introduction

Dopamine is an important transmitter molecule in vertebrate nervous and endocrine tissues whose signaling is transduced by two pharmacologically distinct plasma membrane receptors known as D_1 and D_2 receptors (Spano *et al.*, 1978; Kebabian and Calne, 1979). Dopamine stimulation of the D_1 receptor subtype increases adenylyl cyclase activity while D_2 receptor stimulation reduces it. Other G protein-mediated signaling events at the D_2 receptor include changes in K^+ (Israel *et al.*, 1988; Castelletti *et al.*, 1989) and Ca^{2+} (Taraskevich and Douglas, 1978) ion channel activity and phosphoinositol hydrolysis-coupled changes in $[Ca^{2+}]_i$ homeostasis (Beaudry *et al.*, 1986; Enjalbert *et al.*, 1986). To account for such diverse effects, the evidence from biochemical studies suggests that signal transduction of D_2 receptors is mediated by at least two different G proteins, G_i and G_o (Senogles *et al.*, 1987; Ohara *et al.*, 1988; Elazar *et al.*, 1989) with a possible involvement of receptor heterogeneity (De Keyser *et al.*, 1989).

Profound pathological consequences arise from alterations in dopamine availability at its receptors, particularly at the D_2 receptor. Most conspicuous among these are the motor control abnormalities of Parkinson's disease in which the activity of the nigra-striatal dopaminergic pathway is compromised by the loss of a midbrain population of neurons synthesizing dopamine (for review, see Baldessarini, 1985). A dysfunction in D_2 receptor-mediated signaling from midbrain to limbic and/or cortical regions may contribute to the psychiatric disorder of schizophrenia (Seeman *et al.*, 1976). In the pituitary, lactotroph insensitivity to dopamine results in hyperprolactinemia and infertility (MacLeod *et al.*, 1970; Thorner *et al.*, 1981). The therapeutic effect of drugs altering dopamine activity (e.g. L-DOPA and the neuroleptics) correlates with their ability to influence D_2 receptor activity. Obviously, the existence of the D_2 receptor in areas having disparate neurophysiological correlates (i.e. control of movement, affect, cognition and endocrine homeostasis) motivates the design of functionally more discriminating drugs. Hence, the characterization of the D_2 receptor is a necessary first step.

Recently, a rat brain cDNA was isolated that displayed sequence similarity to members of the G protein-coupled receptor superfamily. Ligand-binding characteristics of the expressed protein showed that the cDNA encodes the rat D_2 receptor (Bunzow *et al.*, 1988). Because of our interest in studying its human homolog, we isolated cognate cDNAs from human pituitary and unexpectedly found a second receptor form differing in the presence of a 29 amino acid residue peptide in a predicted intracellular receptor domain. We describe here the origin of this structural diversity, show that mRNAs encoding the two receptor forms exist in human and rat brain and pituitary, and provide an initial pharmacological profile of the expressed receptors. We speculate that structural heterogeneity of the D_2 receptor permits differential access to the complex metabolic machinery of cells targeted by dopamine.

Results

The human dopamine D_2 receptor

Cloned cDNA encoding the human D_2 receptor was isolated by screening a pituitary cDNA library with oligonucleotides rD_2-5 and rD_2-3 encoding rat D_2 receptor sequences (Bunzow *et al.*, 1988). The cDNA and deduced polypeptide sequence of the human pituitary D_2 receptor is shown in Figure 1. This receptor is 443 amino acid residues long and contains seven transmembrane spanning regions (TM1-TM7) which have come to be recognized as hallmarks of all G protein-coupled receptors (Dohlman *et al.*, 1987). The polypeptide is highly similar to the rat brain D_2 receptor with the notable exception of a 29 residue insertion (peptide X) in a domain between TM5 and TM6. In receptors of similar design this domain is intracellularly located and has been implicated in G protein interaction (Kobilka *et al.*,

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1    CTGGCCACCCAGTCGGTCCACCGCCCTGATGGATCCACTGAATCTGTCCTGGTATGATGA   60
1                              M  D  P  L  N  L  S  W  Y  D  D     11

61   TGATCTGGAGAGGCAGAACTGGAGCCGGCCCTTCAACGGGTCAGACGGGAAGGCGGACAG  120
12    D  L  E  R  Q  N  W  S  R  P  F  N  G  S  D  G  K  A  D  R    31
                                          E

121  ACCCCACTACAACTACTATGCCACACTGCTCACCCTGCTCATCGCTGTCATCGTCTTCGG  180
32    P  H  Y  N  Y  Y  A  T  L  L  T  L  L  I  A  V  I  V  F  G   TM1
                          M                    F  I

181  CAACGTGCTGGTGTGCATGGCTGTGTCCCGCGAGAAGGCGCTGCAGACCACCACCAACTA  240
52    N  V  L  V  C  M  A  V  S  R  E  K  A  L  Q  T  T  T  N  Y   71

241  CCTGATCGTCAGCCTCGCAGTGGCCGACCTCCTCGTCGCCACACTGGTCATGCCCTGGGT  300
72    L  I  V  S  L  A  V  A  D  L  L  V  A  T  L  V  M  P  W  V   TM2

301  TGTCTACCTGGAGGTGGTAGGTGAGTGGAAATTCAGCAGGATTCACTGTGACATCTTCGT  360
92    V  Y  L  E  V  V  G  E  W  K  F  S  R  I  H  C  D  I  F  V   111

361  CACTCTGGACGTCATGATGTGCACGGCGAGCATCCTGAACTTGTGTGCCATCAGCATCGA  420
112   T  L  D  V  M  M  C  T  A  S  I  L  N  L  C  A  I  S  I  D   TM3

421  CAGGTACACAGCTGTGGCCATGCCCATGCTGTACAATACGCGCTACAGCTCCAAGCGCCG  480
132   R  Y  T  A  V  A  M  P  M  L  Y  N  T  R  Y  S  S  K  R  R   151

481  GGTCACCGTCATGATCTCCATCGTCTGGGTCCTGTCCTTCACCATCTCCTGCCCACTCCT  540
152   V  T  V  M  I  S  I  V  W  V  L  S  F  T  I  S  C  P  L  L   TM4
                    A

541  CTTCGGACTCAATAACGCAGACCAGAACGAGTGCATCATTGCCAACCCGGCCTTCGTGGT  600
172   F  G  L  N  N  A  D  Q  N  E  C  I  I  A  N  P  A  F  V  V   191
                    T

601  CTACTCCTCCATCGTCTCCTTCTACGTGCCCTTCATTGTCACCCTGCTGGTCTATATCAA  660
192   Y  S  S  I  V  S  F  Y  V  P  F  I  V  T  L  L  V  Y  I  K   TM5

661  GATCTACATTGTCCTCCGCAGACGCCGCAAGCGAGTCAACACCAAACGCAGCAGCCGAGC  720
212   I  Y  I  V  L  R  R  R  R  K  R  V  N  T  K  R  S  S  R  A   231
                       K

721  TTTCAGGGCCCACCTGAGGGCTCCACTAAAGGGCAACTGTACTCACCCCGAGGACATGAA  780
232   F  R  A  H  L  R  A  P  L  K  G  N  C  T  H  P  E  D  M  K   X
                    N     K  T

781  ACTCTGCACCGTTATCATGAAGTCTAATGGGAGTTTCCCAGTGAACAGGCGGAGAGTGGA  840
252   L  C  T  V  I  M  K  S  N  G  S  F  P  V  N  R  R  R  V  E   271
                                                               D

841  GGCTGCCCGGCGAGCCCAGGAGCTGGAGATGGAGATGCTCTCCAGCACCAGCCCACCCGA  900
272   A  A  R  R  A  Q  E  L  E  M  E  M  L  S  S  T  S  P  P  E   291

901  GAGGACCCGGTACAGCCCCATCCCACCCAGCCACCACCAGCTGACTCTCCCCGACCCGTC  960
292   R  T  R  Y  S  P  I  P  P  S  H  H  Q  L  T  L  P  D  P  S   311

961  CCACCATGGTCTCCACAGCACTCCCGACAGCCCCGCCAAACCAGAGAAGAATGGGCATGC 1020
312   H  H  G  L  H  S  T  P  D  S  P  A  K  P  E  K  N  G  H  A   331
                          N

1021 CAAAGACCACCCCAAGATTGCCAAGATCTTTGAGATCCAGACCATGCCCAATGGCAAAAC 1080
332   K  D  H  P  K  I  A  K  I  F  E  I  Q  T  M  P  N  G  K  T   351
       I  V        R           F

1081 CCGGACCTCCCTCAAGACCATGAGCCGTAGGAAGCTCTCCCAGCAGAAGGAGAAGAAAGC 1140
352   R  T  S  L  K  T  M  S  R  R  K  L  S  Q  Q  K  E  K  K  A   371

1141 CACTCAGATGCTCGCCATTGTTCTCGGCGTGTTCATCATCTGCTGGCTGCCCTTCTTCAT 1200
372   T  Q  M  L  A  I  V  L  G  V  F  I  I  C  W  L  P  F  F  I   TM6

1201 CACACACATCCTGAACATACACTGTGACTGCAACATCCCGCCTGTCCTGTACAGCGCCTT 1260
392   T  H  I  L  N  I  H  C  D  C  N  I  P  P  V  L  Y  S  A  F   411

1261 CACGTGGCTGGGCTATGTCAACAGCGCCGTGAACCCCATCATCTACACCACCTTCAACAT 1320
412   T  W  L  G  Y  V  N  S  A  V  N  P  I  I  Y  T  T  F  N  I   TM7

1321 TGAGTTCCGCAAGGCCTTCCTGAAGATCCTCCACTGCTGACTCTGCTGCCTGCCCGCACA 1380
432   E  F  R  K  A  F  L  K  I  L  H  C  *                       443
                          M

1381 GCAGCCTGCTTCCCACCTCCCTGCCCAGGCCGGCCAGCCTCACCCTTGCGAACCGTGAGC 1440
1441 AGGAAGGCCTGGGTGGATCGGCCTCCTCTTCACCCCGGCAGGCCCTGCAGTGTTCGCTTG 1500
1501 GCTCCATGCTCCTCACTGCCCGCAC...                                  1525
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Fig. 1. The human dopamine D_2 receptor cDNA and polypeptide sequence. The amino acid sequence is presented in the one-letter code and the rat sequence (Bunzow *et al.*, 1988), when different, is indicated below the human sequence. Between positions 332 and 333 of the human polypeptide one residue is inserted in the rat homolog. Proposed polypeptide regions are underlined and listed on the right. The additional 29 residue peptide specified by the cloned human cDNA and not by the published rat sequence is boxed and designated by X. Potential N-linked glycosylation sites are denoted by ovals.

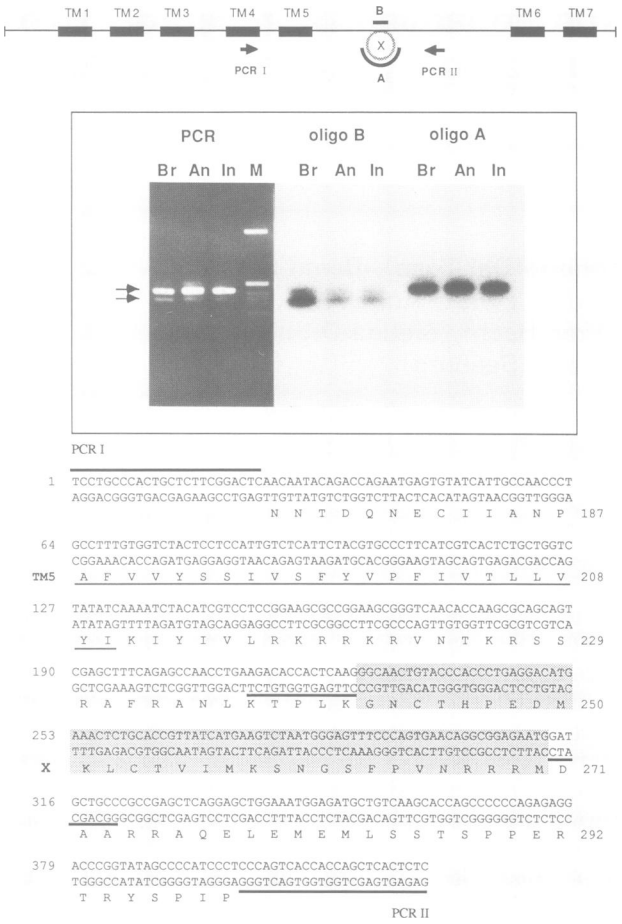

Fig. 2. The two molecular forms of D_2 receptor mRNA detected by the polymerase chain reaction (PCR). A pair of oligonucleotides (PCR I and PCR II), located as indicated in the receptor outline schematically depicted on top, were used as primers on cDNA generated from mRNA of rat brain (Br), pituitary anterior lobe (An) and intermediate lobe (In). The DNA products of these reactions resolved by agarose gel electrophoresis and their hybridization with oligo A, specific for sequences encoding the larger receptor form, and with oligo B, specific for the short receptor form, are shown in the composite middle panel. Arrows point to the 420 bp (upper) and 330 bp (lower) DNA fragments. Fragment lengths were determined relative to *Hin*fI-cleaved pBR322 DNA (M). DNA sequences obtained from the cloned PCR products are also presented. The sequences of the two primers PCR I and PCR II are indicated at the DNA termini. The TM5 region is underlined. The shaded region highlights sequence X only found in cloned DNA derived from the 420 bp PCR-generated DNA. The sequence of oligonucleotide B (23mer) is underlined. It flanks the shaded region X but is contiguous in DNA encoding the rat D_{2B} receptor form.

1988; Kubo *et al.*, 1988). It displays the highest sequence variability between different receptors and their subtypes (Kubo *et al.*, 1986; Peralta *et al.*, 1987) and is subject to variability in different species. Peptide X is not homologous to any known sequence and does not contain obvious phosphorylation consensus sites.

Apart from the inserted sequence, all other features of the human D_2 receptor, notably the absence of a signal sequence, the presence of N-linked glycosylation sites and the conservation of sequences within the seven transmembrane regions relative to other G protein-coupled receptors, are as described for the rat D_2 receptor (Bunzow *et al.*, 1988).

Two D_2 receptor forms exist in rat and man

To investigate the existence of mRNAs encoding the larger form of the rat D_2 receptor, the polymerase chain reaction (PCR) (Mullis and Faloona, 1987) was carried out with template cDNAs constructed with mRNAs of rat brain as well as pituitary anterior and intermediate lobes. A pair of oligonucleotides served as primers of which one (primer PCR I) encoded part of TM4 and the other (primer PCR II) was complementary to a cDNA sequence encoding a peptide located C-terminal to the 29 residue insertion. The gel-resolved DNA products comprised two DNA fragments

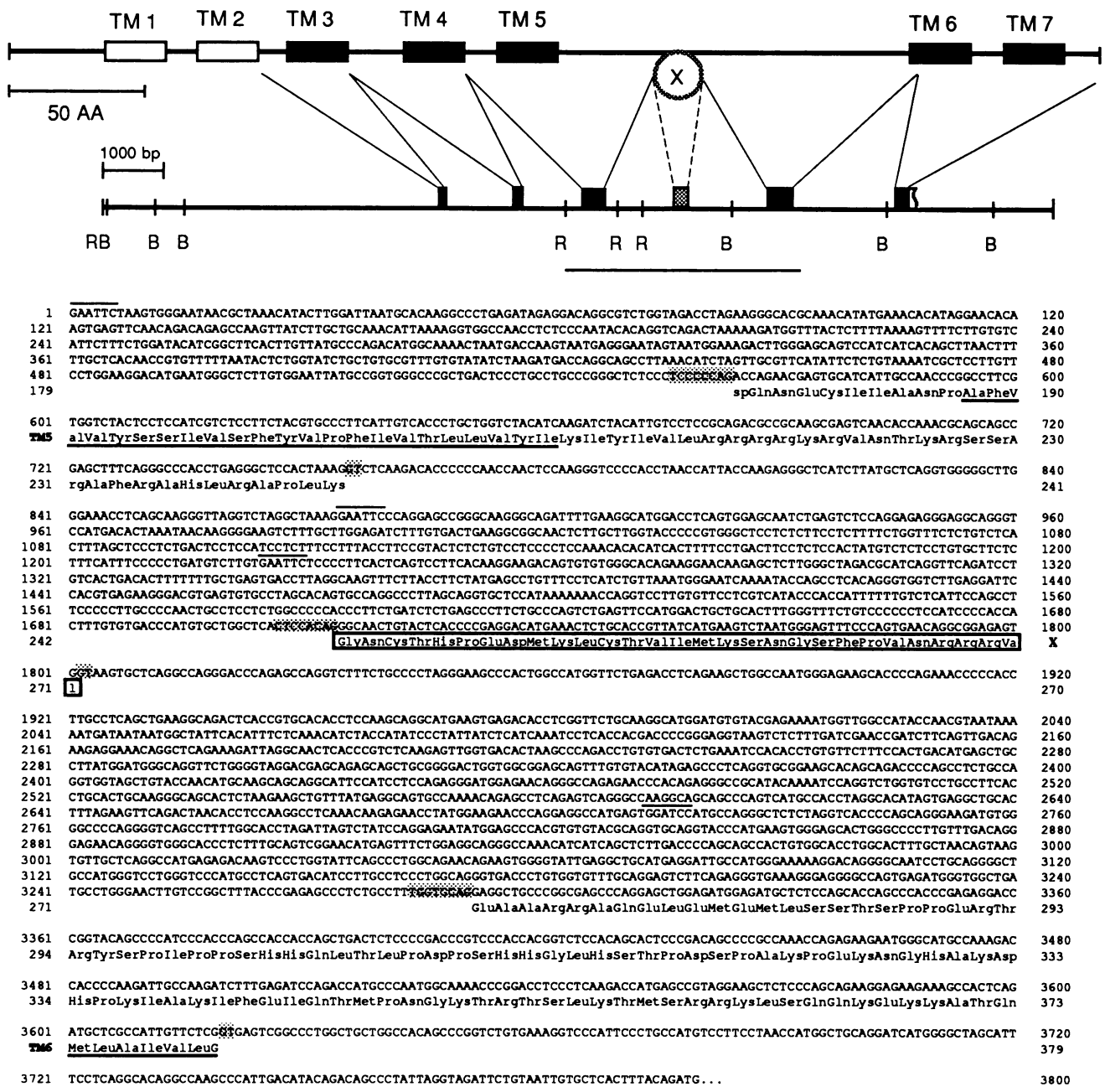

Fig. 3. The human D_2 receptor gene contains a separate exon for sequence X. Shown on top is the arrangement of the proposed transmembrane segments (boxed) and their connecting regions in the receptor. Peptide X is indicated by a shaded loop. Receptor regions are connected by thin lines to exons contained on a gene map shown underneath. The isolated DNA does not contain coding regions for protein sequences N-terminal to, and including, TM2 (open boxes) but probably contains the 3′-untranslated region of the mRNA (open wavy-lined box extending the TM6−TM7 exon). In the gene map, R and B represent all the *Eco*RI and *Bam*HI restriction endonuclease sites respectively. The broken line connecting peptide X to its exon (stippled box) denotes an alternative splice. The DNA sequence presented below the gene map covers the underlined gene region. In this sequence, exon X is boxed, TM5 and the beginning of TM6 are underlined, splice donor and acceptor sites are shaded. For reference, *Eco*RI and *Bam*HI sites are overlined.

~330 and ~420 bp in length (Figure 2), consistent with two rat D_2 receptor mRNA species. Both amplified DNAs originating from rat brain cDNA were gel excised and cloned into M13 vectors for sequence determination. The 420 bp DNA-containing M13 clones specified the rat sequence for the longer D_2 receptor form, while the 330 bp DNA subclones contained the sequence of the published (Bunzow *et al.*, 1988) small form of the rat D_2 receptor (Figure 2). These data document the specificity of the oligonucleotide primers and strongly indicate that both D_2 receptor mRNAs are expressed in the rat.

As judged by the relative abundance of both PCR-generated DNAs in the three tissues investigated, the mRNA encoding the longer receptor form is expressed at 5- to 10-fold higher levels than the mRNA for the smaller form (Figure 2). This differential expression is in accordance with the results of our human pituitary cDNA screen where all cDNA clones analyzed encoded only the larger form (or were incomplete, precluding a distinction between the two sequences). However, both receptor forms also exist in man at similar relative abundances seen in the rat. This was determined by PCR analysis of cDNA constructed from

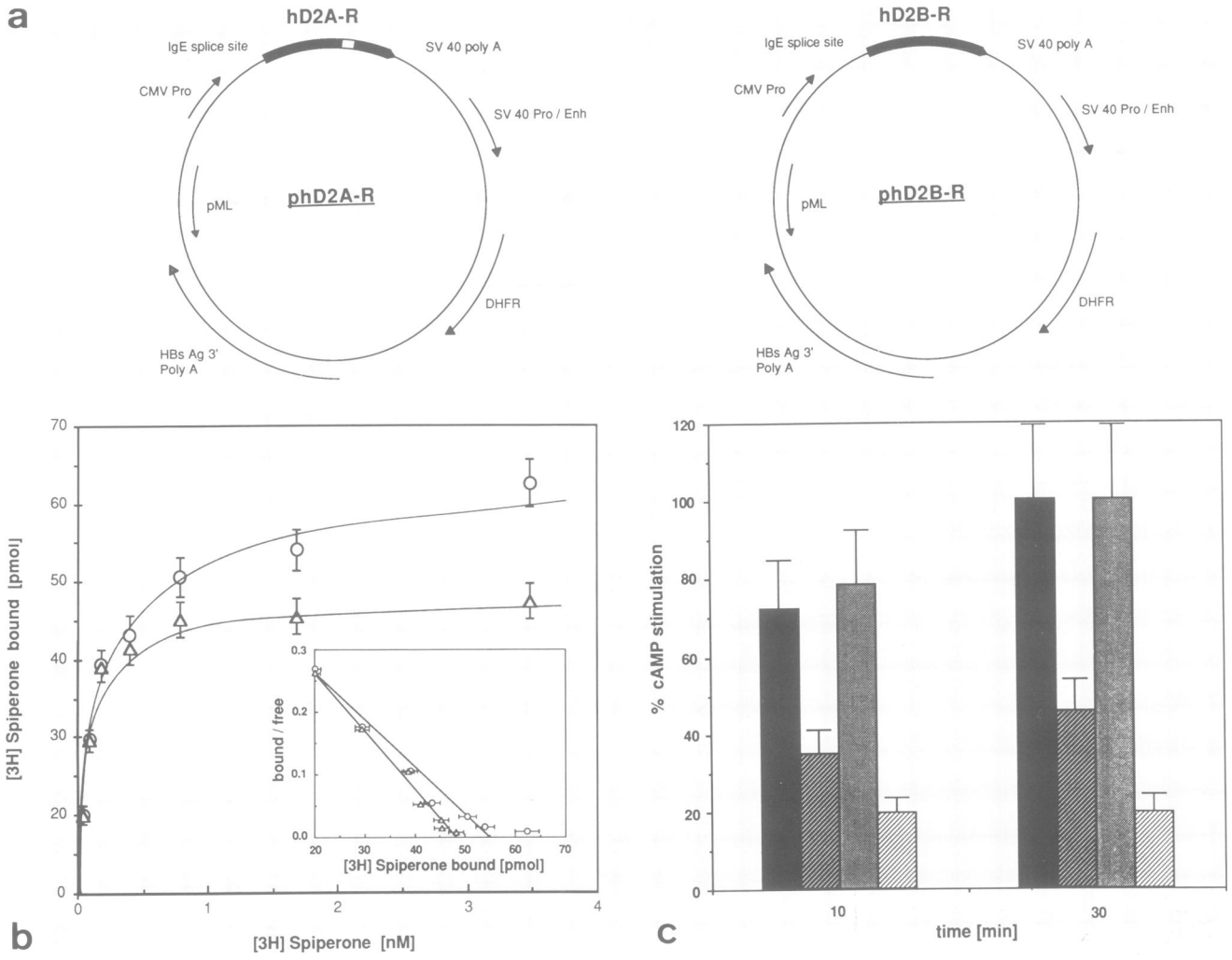

Fig. 4. Functional expression of human dopamine D_2 receptor forms A and B. (**a**) Schematic illustration of expression plasmids phD_{2A}-R and phD_{2B}-R, showing the major functional elements (Gorman *et al.*, 1989). (**b**) Saturation isotherms of D_2 receptor antagonist [^{3}H]spiperone binding to crude membranes prepared from 293 cells transfected with phD_{2A}-R (○) or hD_{2B}-R (Δ). The corresponding Scatchard analyses are shown in the insert. Error bars correspond to values ± SEM from three independent experiments. (**c**) Inhibition of adenylyl cyclase. The bars show normalized cyclic AMP levels measured after 10 or 30 min of agonist incubation of 293 cells transiently co-expressing either of the D_2 receptor forms and the lutropin receptor. D_2 receptor forms and agonists were as follows—black bars hD_{2A}-R, hCG; darkly hatched bars hD_{2A}-R, hCG + DA; stippled bars: hD_{2B}-R, hCG; lightly-hatched bars: hD_{2B}-R, hCG + DA. The maximal cyclic AMP levels obtained after 30 min of hCG stimulation in the absence of dopamine were arbitrarily set to 100%. In three independent experiments these levels were 850 ± 150 fmol cyclic AMP/800 cells for hD_{2A}-R and 500 ± 100 fmol cAMP/800 cells for hD_{2B}-R. Accordingly, the error bars comprise ~20% of all determined cyclic AMP values.

human caudate-putamen RNA using as primers oligonucleotides PCRI and hD5. Two DNA fragments of 390 and 300 bp were generated which showed the expected sequence and hybridization to oligonucleotides specific for X or spanning the X-encoding sequence (not shown). Hence, for both species, we have designated the longer, more prevalent form as D_{2A}-R and the smaller form as D_{2B}-R.

To demonstrate independently that the PCR-generated rat DNA fragments resulted from amplification of the two receptor-encoding cDNAs, the gel resolved DNAs were blotted in duplicate onto nitrocellulose membranes. One blot was hybridized with a 3′-end-labeled 45mer oligonucleotide (oligo A) complementary to the human DNA sequence encoding part of peptide X. The other blot was hybridized with a similarly labeled 23mer oligonucleotide (oligo B) complementary to a contiguous sequence in the rat D_{2B}-R-encoding DNA but interrupted in D_{2A}-R-encoding DNA by peptide X-specific coding sequence. Strict specificity of these probes had been observed with filter-immobilized M13-cloned 420 and 330 bp DNA (not shown). Using the blots, only the 420 bp DNA fragment hybridized with oligo A, whereas oligo B preferentially detected the 330 bp DNA (Figure 2). The residual hybridization of oligo B with the 420 bp DNA is likely to be caused by a small amount of co-migrating single-stranded 330 base DNA. Such single-stranded DNA is known to be generated in PCR reactions (Harbarth and Vosberg, 1988). In fact, the faint oligo A-labeled band on top of the 420 bp DNA probably represents the single-stranded form of this DNA species.

A separate exon encodes sequence X

To trace the origin of the two forms of D_2 receptor mRNA, the cognate gene was isolated from a human genomic library (Lawn *et al.*, 1978). Mapping and partial DNA sequencing of two independently isolated, largely overlapping human DNA segments revealed that the D_2 receptor gene is highly mosaic with many TM-encoding regions located on separate exons (Figure 3). Such an organization is different from that

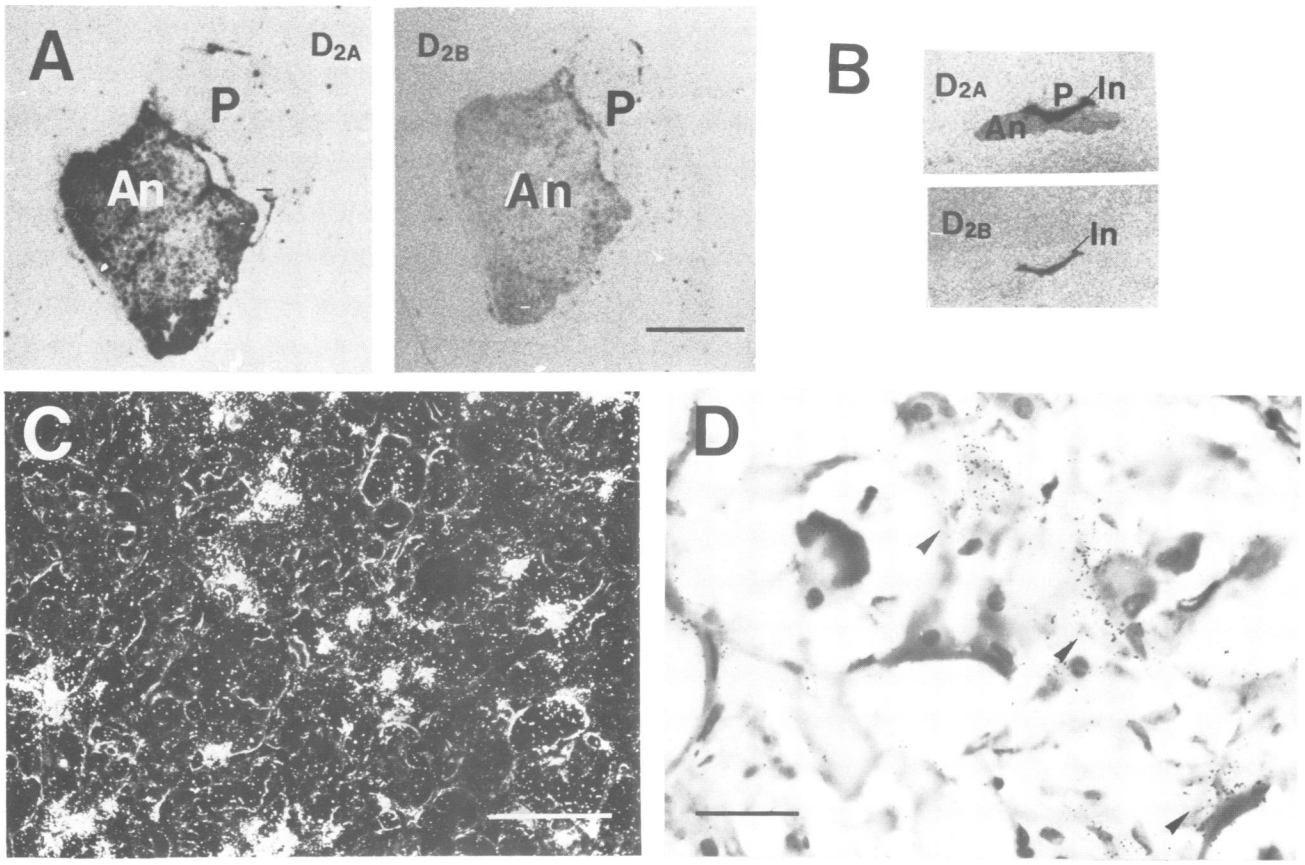

Fig. 5. Autoradiographic detection of cells containing D_{2A} and D_{2B} receptor mRNAs in human and rat pituitaries following *in situ* hybridization with ^{35}S-labeled oligonucleotides. (**A**) and (**B**) are film autoradiograms from sections probed with either oligo A specific for the long (D_{2A}) receptor form or oligo B specific for the short (D_{2B}) form. In human pituitary (**A**), both forms of the D_2 receptor are detected in the anterior (An) but not the posterior (P) lobes with the longer forms present in greater abundance. No distinct intermediate lobe (In) exists in the adult human and this cell type intermingles with cells of the anterior lobe (Halmi, 1983). In rat (**B**), the long and short forms are readily detected in the intermediate lobe with the long form clearly more abundant in both anterior and intermediate lobe than the short form. (**C**) and (**D**) are emulsion autoradiograms from sections of a human pituitary (19 h post-mortem) showing the cellular location of the long (**C**) or short (**D**) form of the D_2 receptor. The hybridization signal appears as collections of white points in dark-field (**C**) or black grains in bright-field (**D**). In (**C**), the longer form is present in a subset of anterior lobe cells. Labeled cells were often clustered around lumina. In (**D**), the shorter form was also present in a subset of anterior lobe cells (arrows), though these cells were less numerous and intensely labeled. Scale bars: A, for A and B, 5 mm; C, 100 μm, D, 50 μm. Exposure times: A, 52 h on β-max fim; B, 20 h on XAR5 film; C and D, 8 days on emulsion.

of many of the other genes for G protein-coupled receptors, notably β-adrenergic and muscarinic receptors where the entire polypeptide is encoded by one large exon (Yarden *et al.*, 1986; Peralta *et al.*, 1987).

Due to the extended nature of the D_2 receptor gene, the isolated human DNA spanning in excess of 15 000 bp does not harbor the entire receptor-coding region. The first exon contained on this DNA encodes the receptor segment corresponding to the entire TM3 region including 13 of the 16 residues between TM2 and TM3. TM2-encoding sequences were not found on the isolated DNA and, as judged from our mapping results, are removed from this exon by >5500 bp of intronic sequence. Five further introns, totaling ~6000 bp, exist between the TM3 exon and the exon encoding the receptor portion from TM6 to the C terminus. The latter exon also specifies 3′-untranslated mRNA sequence and may well contain the entire 3′-non-coding region of the D_2 receptor gene.

The continuous genomic DNA sequence determined across the region encoding the large intracellular loop of the D_2 receptor between TM5 and TM6 showed the presence of two introns (Figure 3). Importantly, these introns flank the coding region for the 29 residue peptide which distinguishes the two receptor forms. The finding of a separate exon for this peptide strongly indicates that alternative splicing is responsible for the existence of the two D_2 receptors.

Both D_2 receptor forms inhibit adenylyl cyclase

The cloned cDNA encoding the human D_{2A} receptor was used for oligonucleotide-mediated deletional mutagenesis (Adelman *et al.*, 1983; Taylor *et al.*, 1985) to generate DNA encoding the smaller receptor form (D_{2B}-R). The DNAs for both D_2 receptors were inserted into a eukaryotic expression vector and the receptors were transiently expressed in human embryonic kidney 293 cells (Gorman *et al.*, 1989). When tested for their ability to bind the dopamine D_2 receptor antagonist [^{3}H[spiperone both receptors displayed indistinguishable, high-affinity binding of this antagonist with a K_D of ~50 pM (Figure 4), consistent with reported values (Seeman, 1981).

We also investigated the effect of D_2 receptor stimulation on adenylyl cyclase activity in transfected 293 cells. To ensure efficient cyclase stimulation the cultured cells were co-transfected with DNA constructed for the expression of the G_s-coupled lutropin/chorionic gonadotropin (hCG) receptor (McFarland *et al.*, 1989). The receptor-expressing

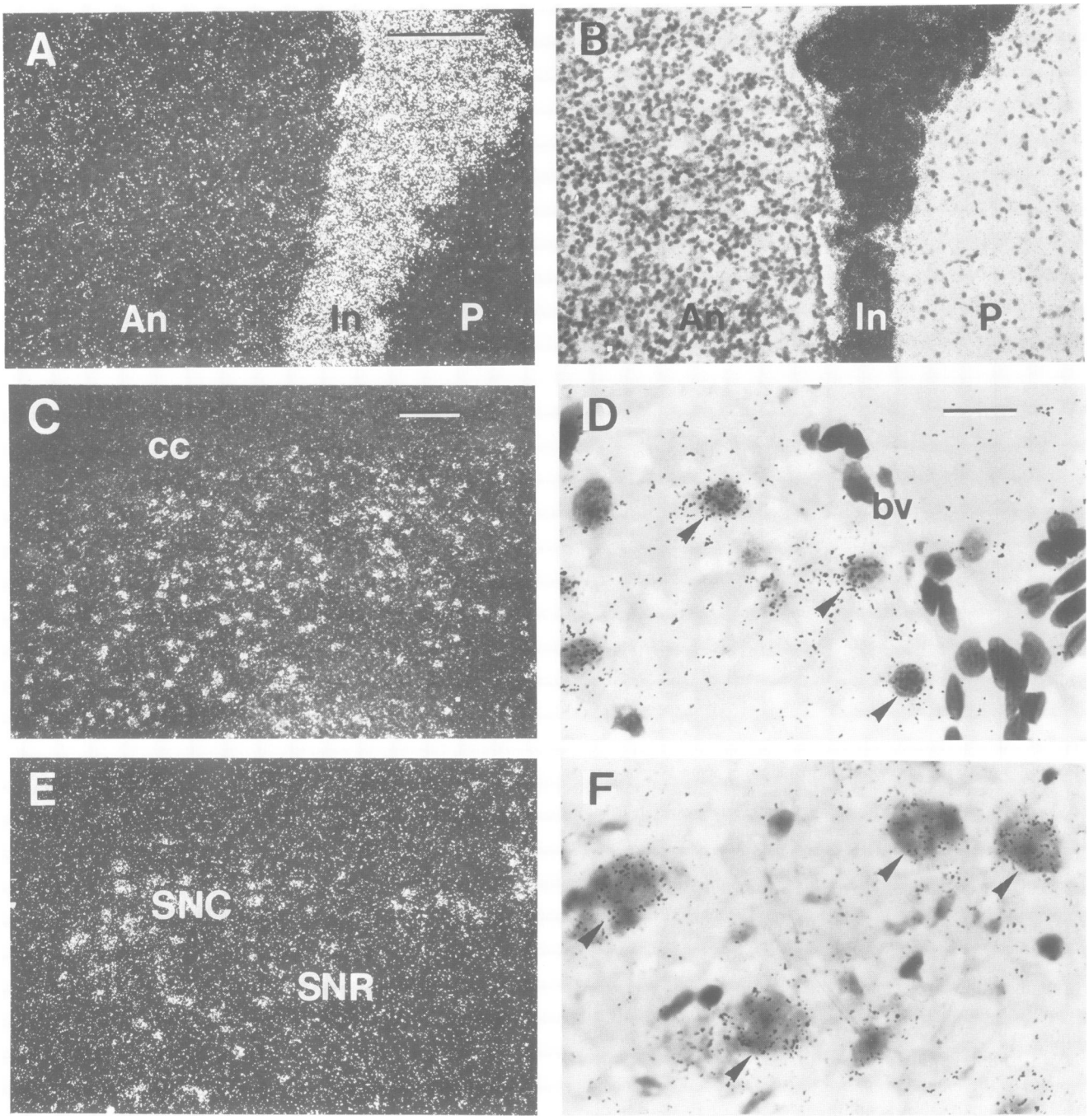

Fig. 6. Autoradiographic detection of cells containing D_{2A} or D_{2B} receptor mRNAs in rat pituitary or brain following *in situ* hybridization with specific ^{35}S-labeled oligonucleotides. **(A)**, **(C)** and **(E)** are dark-field and **(B)**, **(D)** and **(F)** are bright-field photomicrographs. In rat pituitary, both D_{2A} **(A)** and D_{2B} **(B)** receptor mRNAs were highly expressed by melanotrophs in the intermediate lobe (In). To a much lesser extent, D_{2A} receptor mRNA was detected in a large population (presumptive lactotrophs) in the anterior lobe of the pituitary (An). The posterior pituitary (P) did not contain D_2-receptor mRNAs. In the rat caudate-putamen, presented sagittally here **(C,D)**, D_{2A} receptor mRNA was readily detected in numerous cells displaying a neuronal appearance in the Nissl stain (arrowheads). Cellular elements such as epithelial cells lining blood vessels (bv) and presumptive glia in the cortico-fugal fiber tracts and corpus callosum (cc) were not labeled. In the substantia nigra (SN), also shown sagitally (E), D_{2A} receptor mRNA was detected in numerous cells in pars compacta (SNC) but not reticulata (SNR). In the ventral tegmental area (F), a large population of neurons (arrow heads) was also labeled by the D_{2A}-receptor-specific oligomer. D_{2B} receptor mRNA was not seen in brain using the present conditions for *in situ* hybridization. Bar in A (for A and B) = 100 μm; bar in C (for C and E) = 100 μm; bar in D (for D and F) = 20 μm. Exposure times, 8–23 days.

cultured cells were incubated in the presence of either hCG alone, or both hCG and dopamine, and cellular cyclic AMP levels were determined subsequently. As expected, the addition of hCG greatly increased cyclic AMP levels. This increase was markedly inhibited by the additional activation of the human D_2 receptors (Figure 4). Notably, the presence of the smaller receptor form correlated reproducibly with lower cAMP levels, both in the presence and absence of dopamine. This difference could reflect a higher efficiency of G_i protein coupling to the smaller D_2 receptor form than to the larger one, but might also result from higher D_{2B} receptor expression.

Cellular location of the two receptor forms

Cells expressing the long or short forms of the D_2 receptor were identified in human pituitary and rat brain and pituitary by *in situ* hybridization using oligonucleotides which had been 3′-end-labeled with [^{35}S]dATP. D_{2A} or D_{2B} receptor mRNAs were selectively detected with oligo A (a 45mer) or oligo B (a 23mer) respectively, using conditions tested with filter-immobilized M13-cloned rat and human D_2 receptor DNA fragments. Both mRNAs were simultaneously detected using the 51mer rD_2-3 which hybridizes to the coding sequence for the receptor C terminus. An oligomer complementary to the LHRH precursor encoding sequence (Seeburg and Adelman, 1984) did not hybridize to human and rat pituitary (not shown).

In human pituitary, results of film and emulsion autoradiograms indicated the presence of both long and short receptor forms in the anterior lobe (Figure 5A, C, D), with the longer form in greater abundance. In the rat intermediate lobe, the results clearly show that melanotrophs express both D_{2A} and D_{2B} receptor mRNAs (Figure 6A and B). The results on the rat anterior pituitary are less dramatic. Film autoradiograms of pituitary sections probed with oligo A (Figure 5B) or rD_2-3 (not shown) consistently showed a moderately intense signal over the anterior lobe. Using the posterior lobe and the microscopic slide itself to estimate background hybridization, emulsion autoradiograms of these sections showed evidence of numerous, lightly labeled cells in the anterior lobe (Figure 6A), presumably corresponding to lactotrophs.

In contrast to the pituitary intermediate lobe, the presence of D_{2B} receptor mRNA in the anterior lobes or brain could not be convincingly demonstrated, probably reflecting low levels of this message. However, in the rat brain, D_{2A} receptor mRNA was readily detected in neurons located in the caudate-putaman (Figure 6C and D), the nucleus accumbens, the olfactory tubercle, the substantia nigra, pars compacta (Figure 6E) and the ventral tegmental area (Figure 6F).

Discussion

The diverse number of signaling events mediated by the dopamine D_2 receptor has led to the suggestion that subtypes of this receptor exist (De Keyser *et al.*, 1989) and that at least two different G proteins mediate signal transduction at the D_2 receptor (Senogles *et al.*, 1987; Ohara *et al.*, 1988; Elazar *et al.*, 1989). We now report the existence of two forms of this receptor in human and rat generated by alternative splicing. Specifically, these forms differ by a 29 residue peptide sequence located in the predicted intracellular domain between TM5 and TM6. The presence of this peptide characterizes the more abundant, larger form (D_{2A} receptor); its absence defines the rarer, shorter form (D_{2B} receptor).

To our knowledge the use of alternative splicing to generate different molecular forms of a receptor has not been documented in members of the G protein-coupled receptor superfamily. In this family, diversification by gene duplication resulting in receptor variants is the rule. Thus, several neurotransmitters, including dopamine, interact with G protein-coupled receptor subtypes encoded by separate genes (Peralta *et al.*, 1987; Emorine *et al.*, 1989). These subtypes serve to diversify the metabolic action of a particular neurotransmitter on the respective target cells. Our finding of two molecular forms of a receptor subtype produced by alternative splicing highlights a novel mechanism to generate diversity in G protein-coupled receptors. The two D_2 receptors differ in a region shown to be important for G protein coupling in similar receptors (for review see Ross, 1989), suggesting that alternative splicing is used to fine-tune receptor interaction with G_i and G_o proteins. In fact, in our preliminary expression studies, the D_{2B} receptor inhibited adenylyl cyclase activity to a greater extent than did the D_{2A} receptor form, commensurate with the notion that the shorter form more effectively couples to a G_i protein.

The respective physiological contributions of the two receptors may prove elusive using conventional pharmacological approaches. The functional differences created by the peptide insertion in the intracellular loop region may have gone unnoticed in previous studies, given similar ligand affinities for the two receptor forms, an unequal abundance and the plethora of signaling events attributed to the D_2 receptor. Hence, the availability of both D_2 receptors in cloned form is a prerequisite for the elucidation of their full functional spectrum.

Insight into their function could be gained from a differential spatial distribution of the two D_2 receptors. So far, we have not succeeded due to difficulties in localizing the rarer, short receptor form in the brain. In the pituitary, where the D_2 receptor inhibits the release of melanotropin from cells in the intermediate lobe and prolactin from anterior lobe lactotrophs (Baldessarini, 1985), both D_2 receptor forms are highly expressed in melanotrophs. These cells are targets of the tuberoinfundibular dopaminergic neurons located in the basal hypothalamus. A population of cells in the anterior lobe, presumably lactotrophs, were lightly labeled by the D_{2A} receptor oligomer. This is consistent with D_2 receptor autoradiography results using [^{3}H]-spiperone which labeled only the melanotrophs (Palacios and Wamsley, 1984). Hypothalamic dopaminergic neurons supplying the pituitary do not express D_2 receptors, and indeed we failed to detect hybridization signals in these neurons. This is in contrast to other brain regions where dopaminergic neurons express D_2 autoreceptors on dendrites and soma to regulate neuronal firing patterns, and on pre-synaptic terminals to regulate dopamine synthesis and release.

In the brain, the cells expressing a D_{2A} receptor subtype were easily detected in many of the regions known to contain dopamine nerve terminals (Björklund and Lindvall, 1984) and D_2 receptor protein (Palacios and Wamsley, 1984). These included the caudate-putamen, a target of the dopaminergic system originating in the substantia nigra, pars compacta as well as the substantia nigra itself. The caudate-putamen contains D_2 receptors arising from three sources: intrinsic neurons, dopaminergic pre-synaptic terminals from the substantia nigra and glutaminergic pre-synaptic terminals from the cortex (Schwarcz *et al.*, 1978; Murrin *et al.*, 1979). Descending projections from intrinsic neurons of the caudate-putamen do not express the D_2 receptor pre-synaptically, although this receptor is known to exist in the dopaminergic targets in the midbrain (Quik *et al.*, 1979). Therefore, the present results provide unambiguous proof that the long form of the D_2 receptor exists post-synaptically on caudate-putamen neurons as well as on dopaminergic neurons, where it acts as an autoreceptor. A recent study localizing D_2 receptor mRNA made this point prematurely because of the

failure to distinguish between alternative forms (Weiner and Brann, 1989). The other regions where we found neurons expressing the D_{2A} receptor represent post-synaptic targets of mesocortical or mesolimbic projections (e.g. olfactory tubercle, nucleus accumbens) or the dopaminergic neurons themselves (ventral tegmental area neurons). So far, we have failed to detect mRNA in neurons of the pre-frontal or cingulate cortex, in agreement with the findings of Weiner and Brann (1989), although D_2 receptors are known to exist in these regions.

Aberrant expression of, or signal transduction by the D_2 receptor has been proposed to be causally linked to psychosis (Seeman *et al.*, 1976). The present findings give rise to the intriguing possibility that an imbalance between the two receptor forms caused by inappropriate splicing of the primary D_2 receptor gene transcript may contribute to this psychopathological condition.

Materials and methods

Materials

[^{3}H]Spiperone (24 Ci/mmol) was purchased from New England Nuclear. Haloperidol and dopamine were gifts from Janssen Pharmaceuticals. IBMX (3-isobutyl-1-methyl-xanthine) was from Sigma. Human chorionic gonadotropin (hCG) was a gift from Dr Deborah Segaloff. Succinyl-cyclic AMP-antibody was the generous gift of Dr Karl-Norbert Klotz. All oligonucleotides were synthesized with the use of an Applied Biosystems DNA synthesizer under the direction of Dr Michael Nassal.

cDNA cloning

A human pituitary cDNA library (Chen *et al.*, 1989) was screened for human D_2 receptor-encoding cDNA using two 5′-end ^{32}P-labeled oligonucleotides (sp. act. 10^6 c.p.m./pmol). These oligonucleotides (rD_2-5: 5′-ATGGATCCACTGAACCTGTCCTGGTACGATGACGATCTGGAGAGGCAGAAC-3′; and rD_2-3: 5′-GCAGTGCAAGATCTTCATGAAGGCCTTGCGGAACTCGATGTTGAAGGTGGT-3′) contain the first and last (complementary strand) 51 nucleotides of the rat D_2 receptor-coding region (Bunzow *et al.*, 1988). Phage plaques hybridizing with both probes were purified, the cloned cDNA inserted into *Eco*RI-digested M13 mp19 RF-DNA (Yanisch-Perron *et al.*, 1985) and resulting recombinant M13 clones sequence analyzed (Sanger *et al.*, 1977).

Deletional mutagenesis and expression vector constructs

The single-stranded human D_{2A} cDNA cloned into M13mp19 DNA was used as a template for deletional oligonucleotide-mediated DNA synthesis (Adelman *et al.*, 1983) in the presence of *Escherichia coli* DNA polymerase I, Klenow fragment (Boehringer Mannheim) and dATP, dTTP, dGTP and dCTPαS (Taylor *et al.*, 1985). The oligonucleotide hDXΔ (5′-CTCGCCGGGCAGCCTCCTTTAGTGGAGCCCT-3′) was complementary to the sequence encoding the amino acid residues RAPLKEAARR, which are contiguous in the D_{2B} receptor-coding region but interrupted by peptide X coding sequence in the D_{2A} receptor cDNA. The mutant detection system developed by Eckstein and collaborators (Taylor *et al.*, 1985) was employed to minimize screening, using conditions as specified in the protocol accompanying the Amersham *in vitro* mutagenesis kit. Several M13 plaques obtained after exonuclease III digestion and *E.coli* polymerase I- and T4 DNA ligase-mediated repolymerization were analyzed by DNA sequencing (Sanger *et al.*, 1977). For eukaryotic expression, cDNA fragments containing the entire coding regions for the hD_{2A} and hD_{2B} receptors were excised with *Eco*RI and *Pst*I (partial *Pst*I digest) from the respective M13 subclones after double-strand synthesis primed with the lac 17mer (Messing *et al.*, 1981) and Klenow fragment. These DNA fragments were gel purified, given blunt termini using T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates and cloned into *Hpa*I-linearized pCIS vector DNA (Gorman *et al.*, 1989). The *Hpa*I site is located 3′ to the IgE splice acceptor site and cloning into this restriction site ensures the presence of an intron in the 5′-untranslated region of the primary transcription product containing cloned sequences and transcribed from the cytomegalovirus (CMV) promoter on the pCIS vector (Gorman *et al.*, 1989). Recombinant vector plasmids were identified by colony hybridization using as a probe the internally ^{32}P-labeled PCR-amplified cDNA fragment also employed for the human D_2 receptor gene identification (see below). The correct orientation of the cloned cDNAs in the pCIS vector was established by supercoil sequencing (Chen and Seeburg, 1985) of hybridizing colonies using the CMV promoter-specific primer 5′-CTTGAGTGACAATGACATCC-3′.

Polymerase chain reaction

Total RNA was isolated (Cathala *et al.*, 1983) from rat brain, anterior pituitary and pituitary intermediate lobe as well as from human caudate-putamen, and the RNAs were transcribed into cDNA by standard methods (Maniatis *et al.*, 1985) using avian myeloblastosis virus reverse transcriptase (Life Science, St Petersburg, FL). Of each cDNA, 5 ng was used as template in a PCR (Mullis and Faloona, 1987) (20 μl final volume, 400 μM of each deoxynucloside triphosphate, 50 mM KCl, 10 mM Tris−HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.01% gelatin) with 20 pmol each of oligos PCR I (5′-TCCTGCCCACTGCTCTTCGGACTC-3′, encoding rat D_2 receptor residues SCPLLFGL within TM4) and PCR II (5′-GAGAGTGAGCTGGTGGTGACTGGG-3′, complementary to a sequence encoding residues PSHHQLTL located in the rat D_2 receptor 30 residues C-terminal to peptide X) as primers and 2.5 units Ampli-Taq (Cetus, CA). The human brain D_2 receptor DNA sequences were amplified similarly using PCR I and hD-5 (for sequence of hD-5, see next section). Reactions were started at 94°C and performed for 30 cycles (94°C, 0.5 min; 60°C, 0.5 min; 72°C, 1 min) in a thermocycler (Techne Corp., Princeton, NJ). Aliquots (2 μl) of these reactions were resolved on 1.5% agarose gels (Sigma) and the gel alkali-blotted onto two layers of Hybond membrane (Amersham). For the rat sequences one membrane was hybridized with oligo A (5′-GTTCACTGGGAAACTCCCATTAGACTTCATGATAACGGTGCAGAG-3′ complementary to a sequence encoding human and rat peptide X) and the other with oligo B (5′-GGGCAGCATCCTTGAGTGGTGTC-3′, complementary to a sequence contiguous in the rat D_{2B} receptor cDNA). In the case of the human sequences, the longer receptor form was detected by oligo A and the shorter form by hDXΔ and by oligo B. The oligonucleotides had been 3′-end ^{35}S-labeled to a specific activity of 2×10^7 c.p.m./pmol (see below). Hybridization was in 30% formamide, 5 × SSC at 37°C and excess probe was washed from membranes at 65°C in 2 × SSC for 15 min.

D_2 receptor gene isolation and analysis

A human genomic library constructed in λ phage charon 4A (Lawn *et al.*, 1978) was screened for D_2 receptor-coding sequences using the 5′-end ^{32}P-labeled oligonucleotide A (see above, sp. act. 7×10^7 c.p.m./μg) and an internally ^{32}P-labeled DNA fragment (sp. act. 5×10^8 c.p.m./μg) encoding the N-terminal 260 residues of the hD_{2A} receptor. This DNA fragment was generated by PCR from an M13 subclone of the cDNA using as amplification primers oligo A and the lac 17mer primer (Messing *et al.*, 1981). Two independent phages hybridizing with both radiolabeled probes were isolated and the *Eco*RI-digested DNA subcloned into the plasmid vector Bluescript (Stratagene, La Jolla, CA). Subclones were analysed by dual digestion with several restriction endonucleases. Agarose gel-resolved DNA fragments were alkali-blotted onto Hybond N membrane (Amersham) and filters hybridized with 5′-end ^{32}P-labeled oligonucleotides containing coding or complementary cDNA sequences for the human D_2 receptor. For DNA sequence analysis (Sanger *et al.*, 1977) appropriate restriction fragments were subcloned into M13mp19 RF-DNA. In some cases sequences were obtained directly from recombinant Bluescript vectors (Chen and Seeburg, 1985). The following oligonucleotides were used as probes and/or sequencing primers: hD_2-TM2, 5′-GATCGTCAGCCTCGCAGTGGC-3′; hD_2-TM3, 5′-CTTCGTCACTCTGGACGTCAT-3′; hD_2-TM3rc, 5′-GATGCTGATGGCACACAAGTT-3′; hD-1, 5′-GCGAGTCAACACCAAACGCAGC-3′; hD-3, 5′-CTGTACTCACCCCGAGGACATG-3′; hD-4, 5′-GTACACATCCATGCCTTGCAGA-3′; hD-5, 5′-GGCTGTACCGGGTCCTCTCGG-3′; hD-6, 5′-GGCAGCCAGCAGATGATGAACACGC-3′; hD-7, 5′-GTTGCAGTCACAGTGTATGTTC-3′. Oligomers hD_2-TM2, hD_2-TM3 and hD_2-TM3rc originate from the respective transmembrane region-encoding sequences with rc denoting the reverse complement. Oligomers hD-1, hD-3, hD-5, hD-6 and hD-7 encode the following human D_2 receptor amino acid residues: RVNTKRS (C-terminal to TM5), CTHPEDM (within X), PERTRYS (rc, beginning at residue 290), VFIICWLP (rc, TM6) and NIHCDCN (rc, end of TM6). Oligomer hD-4 corresponds to the reverse complement of nucleotides 1987−2008 in the DNA sequence shown in Figure 3.

Binding assay

Exponentially growing 293 cells (4×10^6 cells/10 cm plate) were transfected as described (Pritchett *et al.*, 1988) with the appropriate hD_2-R expression vector (20 μg DNA/10 cm plate). Forty-eight hours after transfection cells were washed twice in PBS (37°C), and harvested by intensive rinsing using ice-cold PBS. Cells were spun (5000 *g*, 10 min), resuspended

in binding buffer (20 mM Tris–HCl, pH 7.4; 120 mM NaCl; 2 mM $MgCl_2$) and homogenized using an Ultrathurax (level 7, 30 s). Homogenates were centrifuged (SW40, 19K, 30 min) and membrane pellets were taken up in the appropriate volume of binding buffer (membranes of 5×10^5 cells transfected with phD_{2A}-R per 2 ml assay volume; 2.5×10^5 cells transfected with phD_{2B}-R). Membranes were incubated for 2 h at 4°C with [^{3}H]spiperone (0.05–3.5 nM) in the presence or absence of 10 μM haloperidol to calculate the amount of specific binding. Membranes were filtered through glass fiber filters (Schleichter & Schuell no. 34) and bound radioactivity determined by liquid scintillation spectrophotometry. Untransfected cells did not show any specific [^{3}H]spiperone binding.

Cyclic AMP assays

293 cells (5×10^5 cells/35 mm plate) were pre-incubated in 1 ml of serum free medium containing 25 mM HEPES, pH 7.2 for 60 min, 48 h after transfection with a mixture of expression plasmids for hD_{2A} or hD_{2B} receptor together with lutropin/hCG receptor. Transfection was as described above. Ten minutes prior to hCG-mediated cyclase stimulation, IBMX (100 mM final concentration) was added to inhibit cyclic AMP phosphodiesterase. Directly before the addition of 20 mM hCG one-half of the cells were incubated with 10 mM dopamine. After 10 and 30 min incubation at 37°C, agonist treatment was stopped by the addition of 200 ml ice-cold 3.9 M perchloric acid. The cells were centrifuged (Eppendorf, 13 000 *g*, 5 min) and the supernatant equilibrated to 0.2 M sodium acetate, pH 6.0, followed by centrifugation (Eppendorf, 13 000 *g*, 5 min). A 250-fold dilution of the clear supernatant into 0.1 M sodium acetate, pH 6.0, was acetylated with triethanolamine and acetonanhydride (both from Sigma) and incubated for 12 h at 4°C in the presence of 5000 c.p.m. [^{125}I]succinyl-cAMP-tyrosine-methylester, anti-cAMP antibody in 0.1 M sodium acetate buffer, pH 6.0, and 0.08% gamma-globulin. After incubation, antibodies were precipitated using 16% PEG (2500 *g*, 20 min, 4°C) and the pelleted radioactivity determined in a gamma counter. Cyclic AMP levels were calculated using a calibration curve determined in parallel by the addition of the cyclic nucleotide in a range between 0 and 5000 fmol.

In situ hybridization

The oligonucleotides A, B and rD_2-3 were 3′-end-labeled to a specific activity of 10^7 c.p.m./pmol ($\sim 10^9/\mu g$). A typical reaction (5 μl, 140 mM cacodylate, pH 7.2, 30 mM Tris base, 2 mM β-mercaptoethanol, 1 mM $CoCl_2$) contained 1 pmol oligonucleotide, 30 pmol of [^{35}S]dATP (NEN, 1100 Ci/mmol) and 5 U terminal deoxynucleotidyl transferase (International Biotechnologies Inc.). Reactions were for 1.5 h at 37°C and terminated by the addition of 50 μl of 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl. Unincorporated [^{35}S]dATP was separated from the labeled oligomer using Biospin 6 chromatography columns (Bio-Rad).

Two adult human pituitaries having post-mortem intervals of 4.5 or 19 h were post-fixed in 4% paraformaldehyde, frozen and cryosectioned at 10 μm. Adult male rat brains and pituitaries were frozen and cryosectioned at 10 μm. All tissues were thaw-mounted onto organosilanized slides. Following fixation for 5 min in 3% neutral-buffered paraformaldehyde containing 0.02% diethyl pyrocarbonate, the sections were rinsed in phosphate-buffered saline, dehydrated in ethanol, air-dried and stored at −70°C. Before hybridization the sections were delipidated in chloroform for 5 min and air-dried. Sections received 50 μl of pre-hybridization buffer for 2 h at 37°C before the addition of hybridization buffer containing the labeled oligonucleotides. Buffers are as described previously (Shivers *et al.*, 1986). Each section received 2×10^5 c.p.m. of labeled probe in 50 μl hybridization buffer which included 4 × SSC, 14 mM β-mercaptoethanol and 50% deionized formamide for oligonucleotides A and rD_2-3, or 30% formamide for oligonucleotide B. The sections were covered with Parafilm and placed in sealed, humidified boxes for overnight hybridization at 37°C. The sections were washed twice in 2 × SSC, 0.05% inorganic pyrophosphate, 14 mM β-mercaptothanol at room temperature for 10 min each and twice in this solution at 65°C for 15 min each. After dehydration and air drying, the sections were exposed to film for 1–3 days and then dipped in Kodak NTB2 emulsion (diluted 1:1 in water) for exposure times of 8–23 days. After autoradiogram development the sections were stained in 2% fast green followed by 0.5% cresyl violet acetate.

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