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Cloning, sequencing and tissue distribution of a candidate G protein-coupled receptor from rat pituitary gland

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A new member of the family of G protein-coupled receptors has been isolated from a rat pituitary cDNA library by the polymerase chain reaction (PCR) using degenerate oligonucleotide primers. The corresponding protein sequence shows seven transmembrane domains and contains conserved regions of homology characteristic of the G protein-coupled class of receptors. The novel receptor mRNA is expressed in the brain, pituitary gland and testis, and has been localized by in situ hybridization in discrete regions of the brain. Expression of the receptor mRNA in *Xenopus* oocytes and in transfected mammalian cells has not yet permitted identification of the corresponding ligand for this receptor.

Polymerase chain reaction; G-protein receptor; Guanine nucleotide-binding regulatory protein; Homology probing

1. INTRODUCTION

Recent developments in receptor biology have shown that a number of the receptors which interact with G protein signalling systems have sequence homologies and may be a part of a receptor superfamily [1]. Specific features of this family include seven stretches of 20–26 residues of hydrophobic amino acids which include several invariant amino acids; potential *N*-linked glycosylation sites near the amino terminus and phosphorylation sites near the carboxy terminus. The presence of partially conserved regions of sequence in the structures of the receptor superfamily permits the use of homology screening in order to isolate novel receptors. We used PCR amplification of cDNA with degenerate oligonucleotide primers corresponding to conserved sequences of various transmembrane domains and have isolated a novel receptor-coding clone from a rat pituitary library. This paper describes the cloning, sequence analysis and tissue expression and localization of this receptor molecule, one of a growing number of G protein- coupled receptors for which ligands have not yet been identified [2–5].

The G-protein coupled receptor cDNA sequence reported here has been assigned the accession number X61496 by the EMBL Data Library.

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2. MATERIALS AND METHODS

2.1. *Rat pituitary cDNA library screens*

Library screens of a size-selected lambda ZAP II cDNA library (10^7 independent clones) prepared from rat pituitary tissue were performed using standard techniques [6]. Bluescript plasmids were rescued from plaque purified clones by M13 excision (Stratagene).

2.2. *Oligonucleotides*

Oligonucleotides were synthesized on an Applied Biosystems PCR-Mate 391A DNA synthesizer according to the manufacturer's instructions. Primer TM3 was based on conserved sequences found in the third transmembrane region [2]; (TM3, 5'GTCGACCTGTG(TC)G (TC)(GC)AT(TC) GCIIT(TG)GA (TC)(AC) G(GC)TAT3'), primer TM6 on those in the sixth transmembrane region; (TM6, 5'AAGCTTA(TG)G(AT)AG(AT)AG GGCAGCCAG CAGAI (GC)(AG)(TC)AAA3'). Primers for analytical PCR were: (matching at 869) 5'TGTAAGAT TGTGATGAGGCACG3' (matching at 1223) 5' GAGAACGCTGCTACACATCG 3'

2.3. *PCR amplification of pituitary cDNA and tissue RNAs*

cDNA reverse transcribed from rat tissue mRNA was submitted to 30 cycles of PCR with Taq polymerase [7]. Cycle conditions were 1.5 min at 93°C, 1 min at 50°C and 2 min at 72°C. PCR was carried out in the presence of [^{32}P]dCTP (2 μCi) for the tissue distribution experiment and the reaction products electrophoresed on a sequencing gel with size standards. Positive and negative controls were run in parallel to check amplification of known sequences and to detect possible template contamination.

2.4. *DNA sequencing and computing*

DNA sequencing was carried out using Sequenase (USB) and analysis was provided by means of a program (GeneJockey) running on Apple Macintosh microcomputers (P.L. Taylor, Biosoft, Cambridge, UK). General database searching and access to the AMT digital array processor (DAP) was obtained via SEQNET (SERC Daresbury Laboratory).

2.5. *In situ hybridization*

Riboprobe preparation from R334 cDNA and in situ hybridization were carried out according to previously described methods [8].

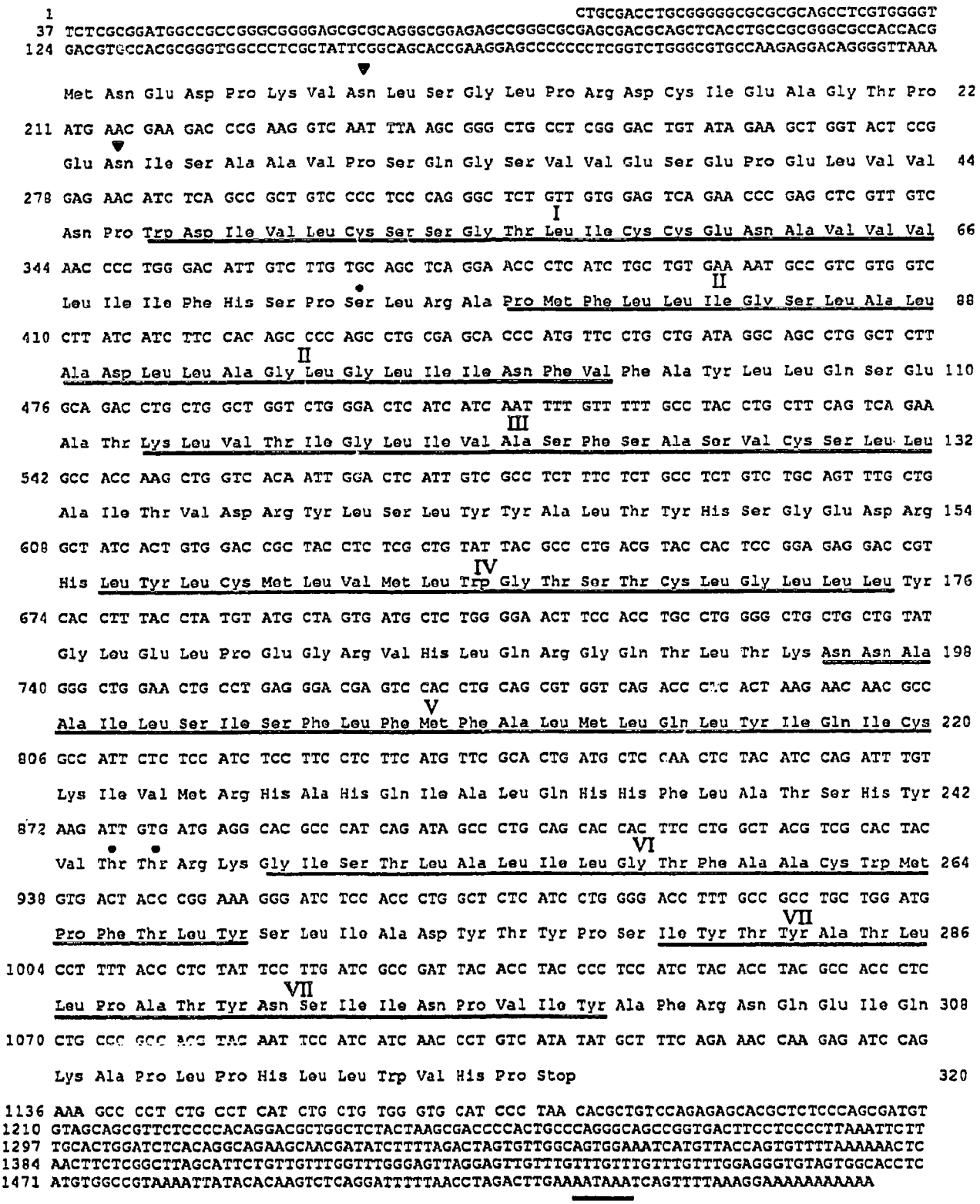

Fig. 1. cDNA and predicted amino acid sequence of the putative R334 receptor. Potential *N*-linked glycosylation sites in the NH_2-terminal region (△), and phosphorylation sites in the COOH-terminal region (●) are indicated. The putative transmembrane domains I–VII are underlined and are assigned on the basis of a Kyte and Doolittle [18] hydropathicity plot. The polyadenylation signal is underlined.

2.6. *Eukaryotic expression*

R334 was cloned into pcDNA1neo vector (Invitrogen) and transfected using Lipofectin Reagent (BRL) into the 293 cell line. G418-selected (Geneticin, Gibco; 800 μg/ml) transfectants were isolated and checked for integration and expression of pcDNA1neo R334 by PCR and Northern blot analysis of cellular mRNA. Cells were maintained

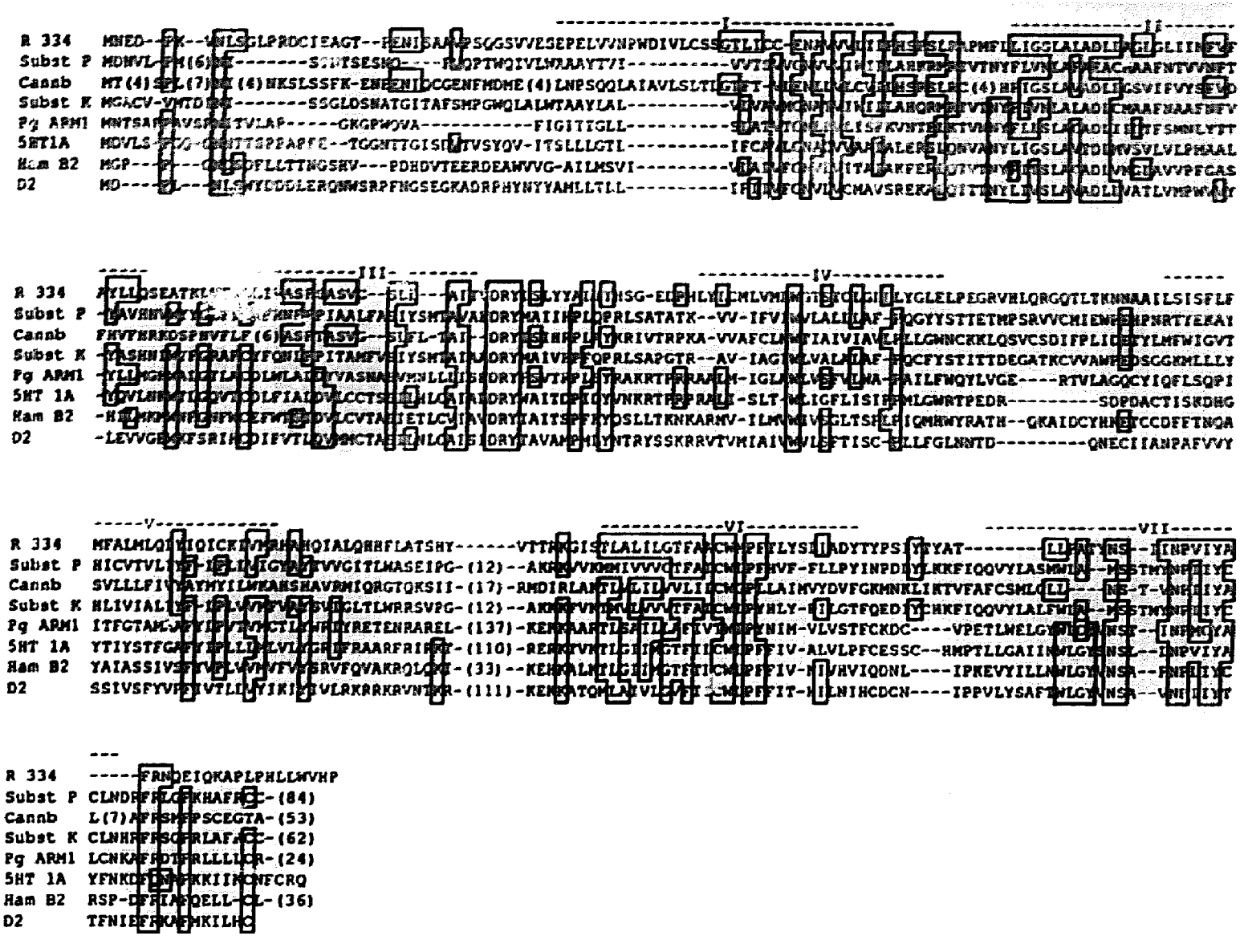

Fig. 2. Alignment of the amino acid sequence encoded by R334 receptor with the amino acid sequences of receptors (rat) for substances P and K, tetrahydrocannabinol, 5HT1A, dopamine D2, acetylcholine (pig muscarinic M1) and adrenaline (hamster β2). The approximate positions of the transmembrane regions are indicated by a broken line above the sequences and conserved residues are boxed.

in DMEM media containing 10% (v/v) heat inactivated foetal calf serum, glutamine (0.3 mg/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml) and incubated at 37°C in 5% CO_2. cAMP was measured [9,10] as were total inositol phosphates (IP1, IP2 and IP3) after extraction and separation [11]. Whole-cell current measurements in *Xenopus* oocytes injected with R334 mRNA transcripts were carried out using two-electrode voltage-clamp techniques [12].

3. RESULTS AND DISCUSSION

PCR products generated from rat pituitary cDNA included a 400 bp band of PCR-amplified DNA which was purified by agarose gel electrophoresis and used to probe a rat pituitary cDNA library. Several independent cDNAs were isolated at low abundance and sequence analysis of these clones showed them to consist of identical stretches of sequence. The most complete cDNA clone, R334, of 1552 bp, contained a region of open reading frame corresponding to a peptide of 320 amino acids (Fig. 1), possessing seven highly hydrophobic regions of 20–25 amino acids. A search was performed on the OWL database for the protein sequence using the PROSRCH program of Coulson et al. [13] running on the DAP. This yielded a list of matching proteins, of which the best-fitting 50 included 47 which were G protein-coupled receptors. None of these proteins were identical with R334, or sufficiently close in structure to suggest membership of a known subfamily. The deduced amino acid sequence of R334 and alignment with 7 other members of the G protein-coupled receptor family is illustrated in Fig. 2.

The R334 sequence contains potential *N*-linked glycosylation sites Asn^{8} and Asn^{24} in the amino terminal region and potential phosphorylation sites, Thr^{244}, Thr^{245} and Ser^{74} in the carboxyl-terminal region. The Asp residue in TM3, shown to be essential for ligand binding in the β-AR receptor [14], is absent in R334 (Fig. 2). Of all the cloned receptors aligned in Fig. 2, R334 contains the shortest third cytoplasmic loop. This loop has been shown to be important for G protein recognition [14]. R334 displays 22% homology across the TM domains with the cannabinoid receptor, 16% and 17% with rat substance P and substance K receptors, 17% with the human β2 adrenergic receptor and 18% with the 5HT1A receptor. The 3'-untranslated region contains a polyadenylation signal and a poly(A) tract (Fig. 1).

The tissue distribution of R334 mRNA in rat tissues was examined. Northern blotting showed a faint hybridizing band greater than 7 kb in brain and testis (not shown). Because Northern analysis was found to be too insensitive to satisfactorily detect the low-level expression of R334 mRNA in these tissues, PCR analysis of reverse-transcribed RNA prepared from pituitary, brain and peripheral tissues was carried out [16]. A PCR-amplified band exactly corresponding to the distance between the two primers as predicted from the R334 sequence (355 bp) was observed in the pituitary gland, brain and testis RNA (Fig. 3). No signal was observed in any of the other tissues examined (see Fig. 3).

The localization and distribution of R334 mRNA in the pituitary gland and brain were studied by in situ hybridization. R334 mRNA was detected in pituitary tissue (Fig. 4A,B), in the piriform cortex of the brain (Fig. 4C,D) as well as in the lateral septal nuclei (not shown). Diffuse labelling of cells was seen in the anterior pituitary gland and posterior lobes (Fig. 4A), while in the brain, the signal was restricted to discrete areas only (Fig. 4C). Control sections probed with the sense riboprobe showed non-specific background hybridization only (Fig. 4B,D).

We have investigated a number of possible ligands for

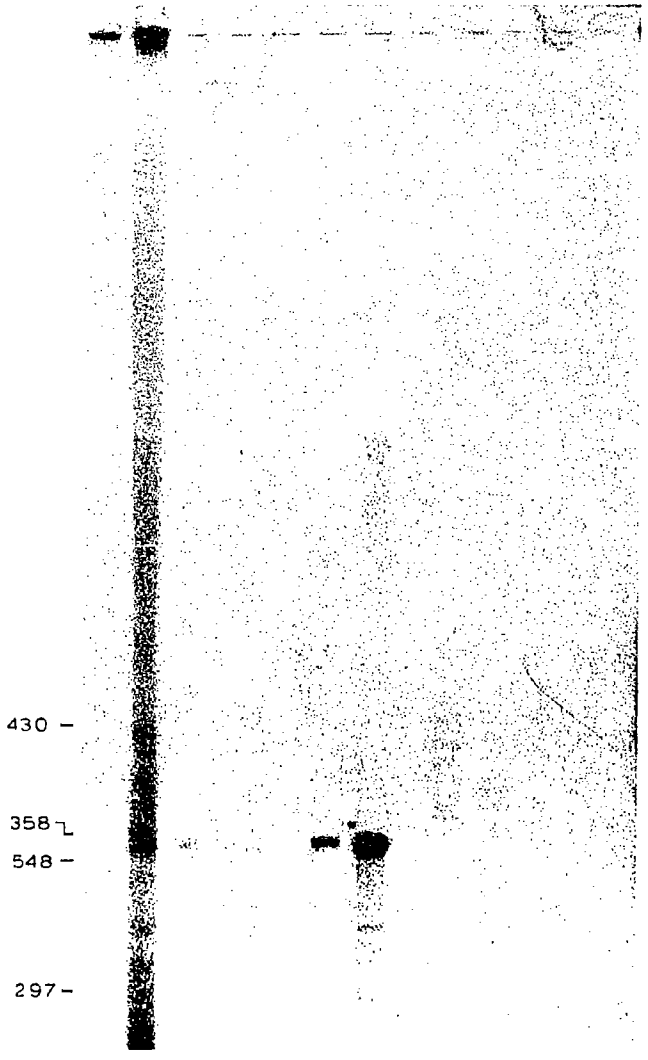

Fig. 3. PCR analysis of R334 transcript tissue distribution. PCR with primers corresponding to R334 was carried out in the presence of [^{32}P]dCTP on cDNAs synthesized from rat tissue RNA. R334 cDNA was employed as a positive control. Labelled reaction products were electrophoresed on a sequencing gel and autoradiographed. From left to right; Lane 1, no DNA; lane 2, R334 cDNA; lanes 3–12, RNA from testis, lung, ovary, pituitary, brain, kidney, liver, spleen, adrenal and heart.

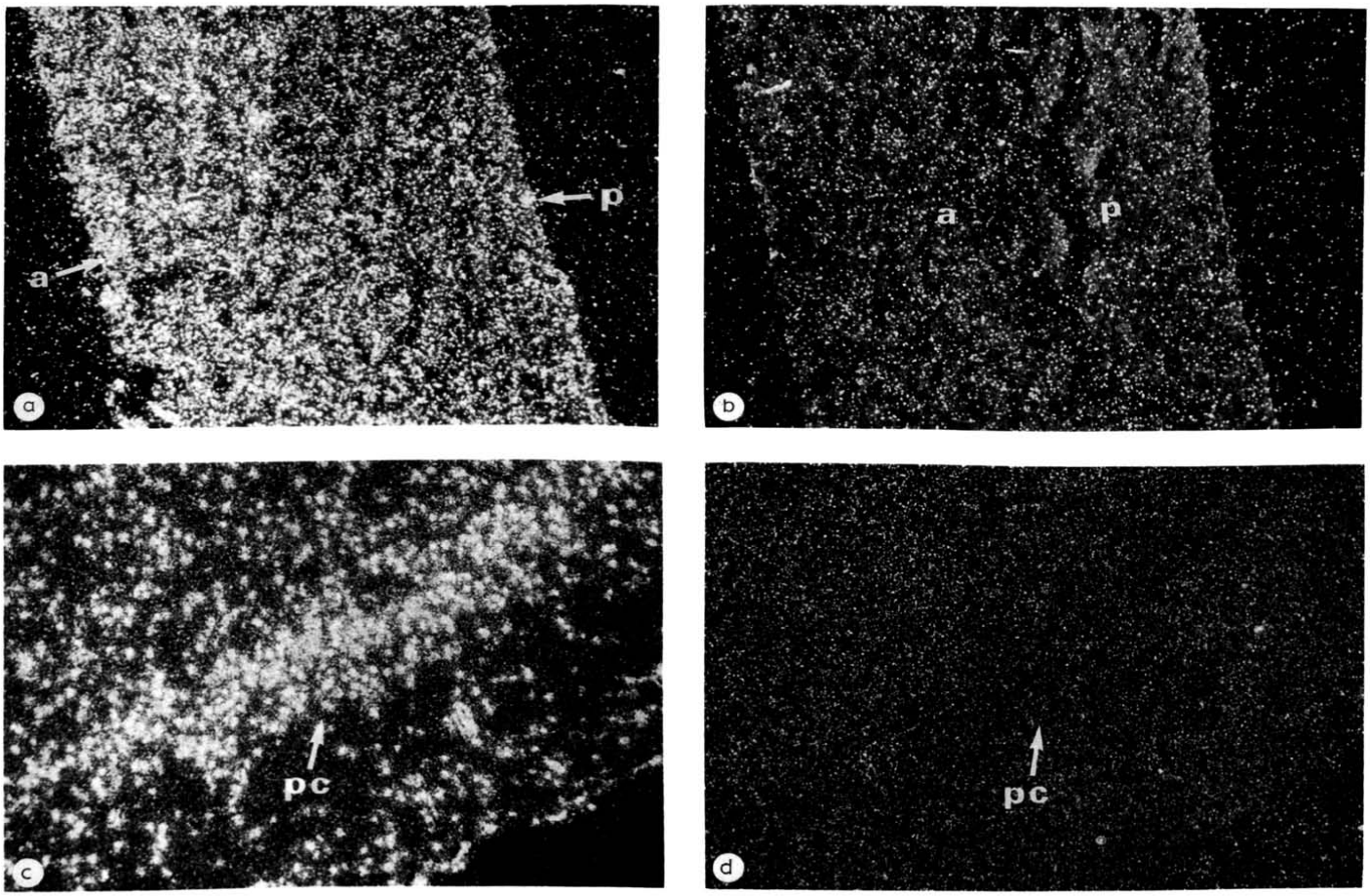

Fig. 4. Autoradiographic detection of cells containing R334 mRNA in rat pituitary and brain tissue by in situ hybridization. Dark-field micrographs of emulsion dipped (A) pituitary horizontal sections (a, anterior; p, posterior), hybridized with R334 antisense riboprobe; (B) pituitary with sense riboprobe; (C) coronal sections through rat brain piriform cortex (pc) hybridized with antisense riboprobe; (D) sense riboprobe.

this receptor. Synthetic, capped RNA transcribed from R334 cDNA was injected into *Xenopus laevis* oocytes, along with RNA transcribed from the 5HT1c receptor cDNA clone [17] as a positive control. 33 different potential ligands were applied to the bathing solution. Although definite electrophysiological responses to serotonin were detected (current = 500–1000 nA), no significant response to any of the drugs was apparent. cAMP and total inositol phosphate assays were also performed on stable transfected cell lines expressing R334 mRNA following exposure to these drugs. None of the substances tested stimulated cAMP production (or inhibited cAMP production in forskolin-treated cells). Similarly, measurements of total inositol phosphates showed no measurable effects (data not shown).

Although we were unable to identify the ligand for the R334 receptor, the observed homologies with known receptor sequences and the characteristics of the hydropathicity profile all suggest strongly that this sequence is that of a previously unknown receptor belonging to the G protein-coupled receptor family. Further studies are required to demonstrate that this molecule functions as a biologically active receptor.

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