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Molecular cloning of a human gene (S31) encoding a novel serotonin receptor mediating inhibition of adenylyl cyclase

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We report the molecular cloning of human gene (S31) containing an open reading frame of 1095 nucleotides, which encodes a protein of 365 amino acids. The encoded protein contains seven hydrophobic putative transmembrane domains considered the hallmark of G protein-coupled receptors. The amino acid sequence shows highest homology to receptors for serotonin (5-hydroxytryptamine). Expression of this receptor in murine L*tk*$^-$ cells conferred upon these cells the ability to respond to serotonin by inhibition of adenylyl cyclase. No response was observed to isoproterenol, epinephrine, histamine, dopamine or melatonin in the transfected cells. We propose that the human gene S31 encodes a novel serotonin receptor.

Serotonin; 5-Hydroxytryptamine; Receptor (human gene); Adenylyl cyclase; Inhibition

1. INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) mediates a wide variety of effects, both in the central nervous system and in the periphery, through several different receptors, of which at least eight discrete subtypes have been proposed based on pharmacological criteria [1]. It is not yet known whether all these pharmacologically distinguishable receptor subtypes are the products of as many different genes, or whether some of the pharmacological differences may arise from tissue-specific factors such as posttranslational modification or association with one or more distinct G proteins. Molecular cloning techniques are now being employed to resolve these questions, and have to date resulted in the cloning of five distinct G protein-coupled mammalian serotonin receptors: 5-HT_{1A} ('G-21') [2,3], 5-HT_{1C} [4], 5-HT_2 [5,6], and two genetically distinct subtypes of the 5-HT_{1D} receptor [7,8]. The human 5-HT_{1D} receptor sequence reported by Hamblin and Metcalf [7] may represent the human homologue of the canine serotonin receptor-like sequence RDC4 reported by Libert et al. [9]. The cloning of a human 5-HT_{1D}-like receptor has also been announced by Zgombick et al. [10], but no structural information has been given. Additionally, a structurally related serotonin receptor has been cloned from *Drosophila* [11].

Employing a low-stringency screening approach, we now report the molecular cloning of another genetically distinct human serotonin receptor, which we refer to as S31. When expressed in murine L*tk*$^-$ cells, this serotonin receptor is shown to mediate inhibition of adenylyl cyclase.

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Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; kb, kilobase(s); MEMα, minimal essential medium, alpha-modified; nt, nucleotide(s); ORF, open reading frame; SDS, sodium dodecylsulphate; *Solutions*: BSS, 126 mM NaCl, 0.64 mM KCl, 98 μM $MgCl_2$, 5 μM $CaCl_2$ and 14.5 mM Tris-HCl, pH 7.5; 1 × Denhardt's solution: 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin; 1 × SSC: 150 mM NaCl and 15 mM sodium citrate.

2. MATERIALS AND METHODS

2.1. *Oligonucleotides*

Oligonucleotides used as screening probes and sequencing primers, up to 69 nucleotides (nt) long, were synthesized on an Applied Biosystems Model 391 DNA Synthesizer using automated cyanoethylphosphoramidite chemistry and prepared for use as described [8]. Oligonucleotide probe A was nt 343–411 of the sense strand of the human 5-HT_{1A} receptor [2]; probe B was nt 400–468 of the sense strand of the rat 5-HT_{1C} receptor [4].

2.2. *Screening, initial analysis, subcloning, and sequencing*

An amplified human *lambda*EMBL3 genomic library (a gift from Dr. David Nelson, Department of Molecular Genetics, Baylor College of Medicine) was screened at low stringency with oligonucleotide probes A and B, as follows. Replicate filters with amplified recombinant phage DNA were prehybridized for 3 h at 42°C in a solution containing 5 × Denhardt's solution, 6 × SSC, 0.5% SDS, 100 μg/ml sheared salmon sperm DNA and 0.3 M Na-phosphate buffer pH 7.0. Hybridization was carried out for 16 h at 42°C in the same solution plus 0.5 × 10^6 cpm/ml each of probe A and probe B labeled at their 5′ end with ^{32}P [12]. The filters were then rinsed three times with 2 × SSC/0.1% SDS at room temperature, then washed for 30 min at 60°C

in 2 × SSC/0.5% SDS, and finally for 30 min at 60°C in 2 × SSC/0.1% SDS. The filters were dried and exposed to Kodak X-Omat AR films with two intensifying screens at −70°C for 5 days (primary screening) or shorter. The high-stringency screening with DNA fragments labeled by nick translation (0.2×10^9 cpm/ml) [12] differed in that after the initial rinsings at room temperature, the filters were subjected to 30 min washes at 68°C, first with 0.2 × SSC/0.5% SDS and then with 0.2 × SSC/0.1% SDS. DNA was prepared from plaque-purified hybridization-positive phages as described [8], and the general strategies followed for the isolation of DNA fragments with partial and full length ORF's and their DNA sequence determination is outlined in Fig. 1A. Briefly, DNA from plaque purified recombinant phages was digested with several restriction enzymes, the digests were electrophoresed in 0.7% agarose and fragments that hybridized to probes A or B were identified by Southern blotting [13]. These fragments were then isolated in preparative scale, purified using Geneclean (BIO 101), and subcloned into plasmid Bluescript KS(+) (Stratagene). After amplification, both strands of the subcloned DNA inserts were directly sequenced by the dideoxy chain termination method of Sanger et al. [14], using alkali-denatured plasmid DNA as template.

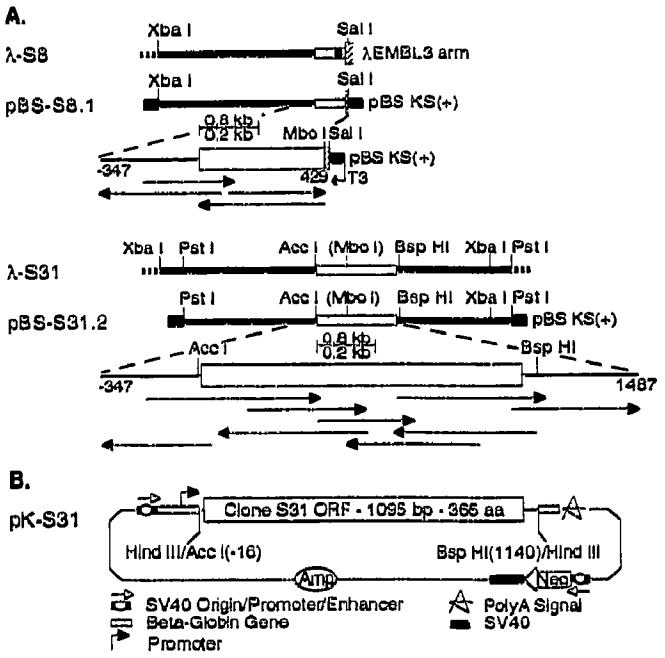

Fig. 1. (A) Summary of molecular cloning and sequencing strategies used to characterize the open reading frame of the human S31 gene. (B) Scheme of the pKNH expression vector after insertion of S31 ORF. ORF's are represented as open boxes, flanking sequences are represented as blank lines connected to ORF's; location of the sequence that corresponds to probes A and B is indicated by the black box within the ORF of *lambda*-S8; *lambda*EMBL3 sequences are represented as hatched boxes, and Bluescript KS(+) sequences are represented as closed boxes at end of inserts. The T3 promoter of Bluescript KS(+) is shown for orientation purposes. Arrows: summary of sequencing runs in which the beginning of the arrows denotes the first readable nucleotide after the sequencing primers and the length of the arrows describes the number of readable nucleotides obtained with each primer. The 0.8-kb markers correspond to representations of phage and total Bluescript inserts; the 0.2-kb markers correspond to amplified regions of Bluescript inserts and the sequencing arrows aligned beneath them. Nucleotide numbering refers to human DNA sequences in Bluescript, where the A of the initiator ATG is nt 1. Plasmid pK-S31 is the expression plasmid obtained upon subcloning the *Acc*I/*Bsp*HI fragment containing the ORF of pBS-S31.2 into pKNH.

2.3. *Subcloning of the full-length ORF into the eukaryotic expression vector pKNH*

pKNH, a kind gift from Prof. S. Numa, Kyoto, Japan, contains two SV40 origin/promoter/enhancer sequences, one directing the expression of the Neomycin resistance gene, the other directing the expression of ORF's cloned into its unique *Hin*dIII site (Fig. 1B, for details see [15]). A fragment containing the ORF derived from *lambda*-S31 was excised from Bluescript DNA, isolated by 0.7% agarose gel electrophoresis, cleaned using Geneclean, rendered blunt-ended with Klenow fragment of DNA polymerase I, and subcloned into *Hin*dIII-digested, blunt-ended and dephosphorylated pKNH vector.

2.4. *Transfection of eukaryotic expression vector pKNH with putative serotonin receptor insert into murine Ltk⁻ cells, and identification and isolation of transformed cell clones*

Mouse Ltk⁻(α-HT) cells (a gift from Dr. Frank Ruddle, Dept. of Biology, Yale University) were cultured, transfected and selected as described previously [16–18].

2.5. *Drugs*

L(−)Isoproterenol (Iso), L(−)epinephrine, serotonin (5-HT), dopamine, histamine and melatonin were from Sigma Chemical Company (St. Louis, MO). Forskolin was from Calbiochem (San Diego, CA).

3. RESULTS AND DISCUSSION

3.1. *Cloning of a full-length ORF from a human genomic library*

In order to clone a novel serotonin receptor we screened at low stringency ca. 1×10^6 recombinant *lambda*EMBL3 phages from a human genomic DNA library that had been amplified twice, using as probe a mixture of two 69-nt long oligonucleotides (probes A and B, see under oligonucleotides above). One of the plaque-purified positive clones, S8, contained a partial ORF that showed sequence homology to 5-HT receptors, but by comparison, appeared to be a novel type. Fig. 1A presents the steps taken to characterize this insert. Clone S8 contained a partial ORF of 429 nt, located at the 3′ end of a 2.5-kb *Xba*I/*Sal*I fragment adjacent to *lambda* DNA. Both strands of this ORF were sequenced fully in Bluescript KS(+) as shown. The whole *Xba*I/*Sal*I fragment was nick-translated and used to screen the same genomic library at high stringency. Four recombinant phages were plaque purified. Restriction mapping indicated that three were derived from the same cloning event as *lambda*-S8. The fourth, *lambda*-S31, descended from a second cloning event. A 4.4-kb *Pst*I/*Pst*I fragment was subcloned into Bluescript KS(+), to give pBS-S31.2, and sequenced as shown in Fig. 1A. Its nucleotide composition was identical to that of pBS-S8.1 except that it contained a complete ORF of 1095 nt. The nucleotide sequence and predicted amino acid composition of the putative serotonin receptor encoded in clone S31 are shown in Fig. 2.

3.2. *Identification of clone S31 as a G protein-coupled serotonin receptor*

The deduced amino acid sequence of the longest open reading frame present in the recombinant DNA frag-

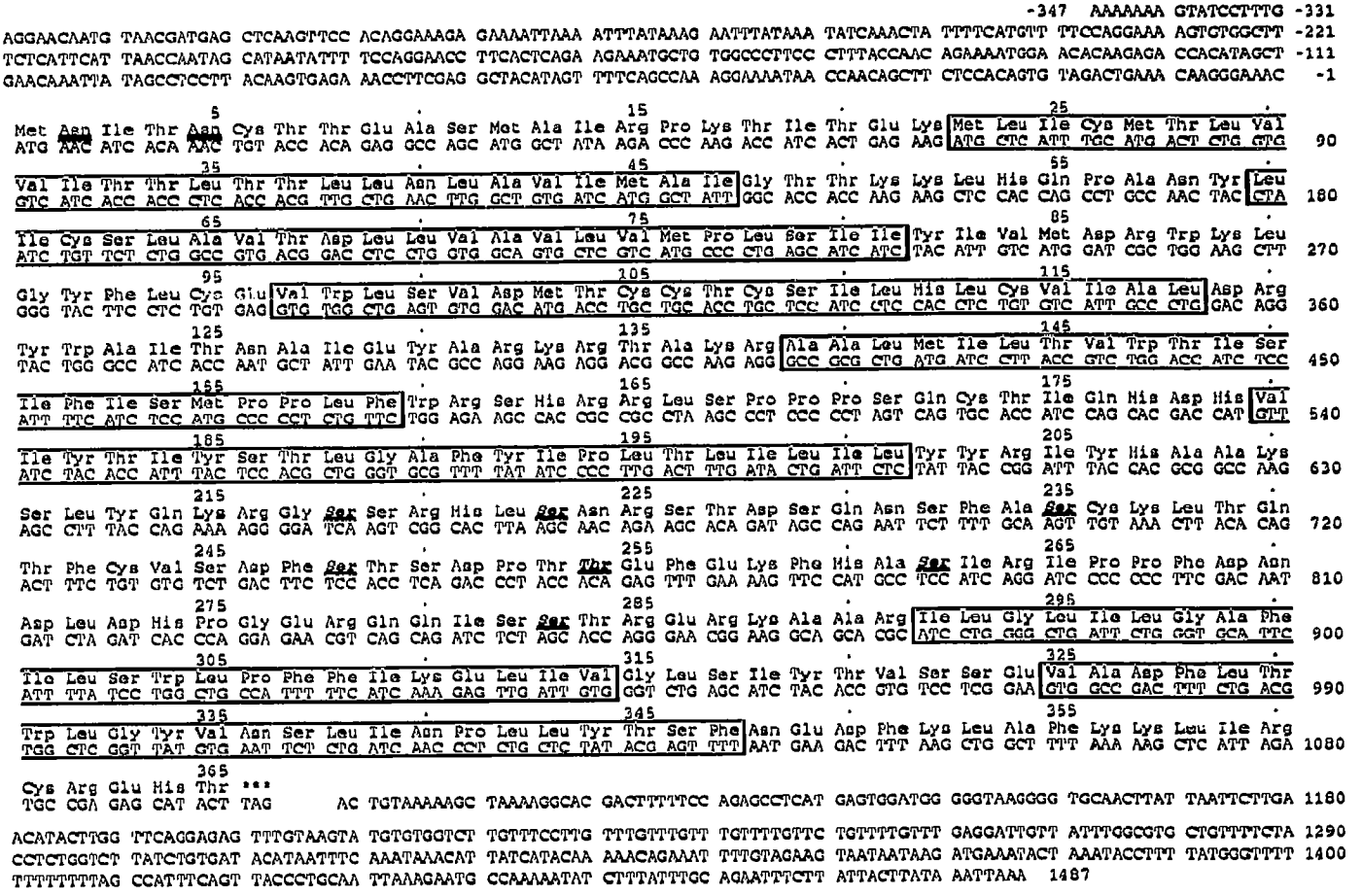

Fig. 2. Nucleotide sequence and deduced amino-acid sequence of human DNA containing the ORF encoding the S31 receptor. Sequences corresponding to the putative seven transmembrane regions are boxed. The two potential asparagine-linked glycosylation sites in the N-terminal extracellular domain are indicated with double underline. Serine and threonine residues located in consensus sequences for phosphorylation by cAMP-dependent protein kinases, protein kinases C or casein kinase II are indicated in bolded italics with single underline. The nucleotide sequence has been submitted to the EMBL Data Library (accession no. Z11166).

ment recognized by probes A/B in *lambda*EMBL3 clone S31, was analyzed for its hydropathy profile and tested for similarity to G protein-coupled receptors, especially serotonin receptors. As indicated by both these analyses (Figs. 3 and 4), the deduced sequence had the landmarks of a G protein-coupled receptor (for review, see [19]): (1) it exhibited seven stretches of hydrophobicity encompassing ca. 20 amino acids, of which six stretches reached hydropathy indices of 2 and the last was less clearly circumscribed (Fig. 3); and (2) it showed extensive relatedness to other G protein-coupled receptors (shown for serotonin receptors in Fig. 4 and Table II). In both Figs. 2 and 4, the most likely stretches of amino acids involved in spanning the plasma membrane are highlighted (as boxes in Fig. 2 and by asterisks in Fig. 4). The ATG assigned as the start codon is the first ATG downstream of an in-frame stop codon (TGA in position −15 to −13) and conforms to the requirement of having a purine in position −3 suggested by Kozak [20]. The very short N-terminal, extracellular end of the predicted amino acid sequence contains two consensus asparagine-linked glycosylation sites (Asp-2 and Asp-5). The putative third intracellular domain is approximately 90 amino acids long and contains one consensus cAMP-dependent protein kinase site (Ser-218), five consensus protein kinase C sites (Serine residues in positions 218, 223, 235, 262, and 283), and three consensus casein kinase II phosphorylation sites (Ser-248, Thr-254, and Ser-283) [21,22]. The intracellular carboxyl-

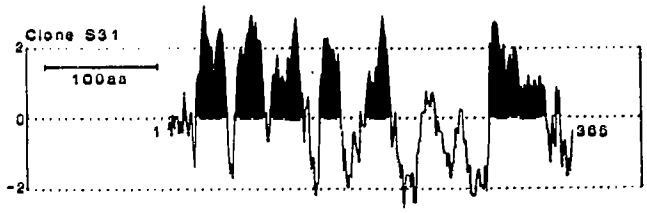

Fig. 3. Hydropathy plot for the protein encoded in clone S31 calculated according to Kyte and Doolittle [25] with a moving window of nine amino acids.

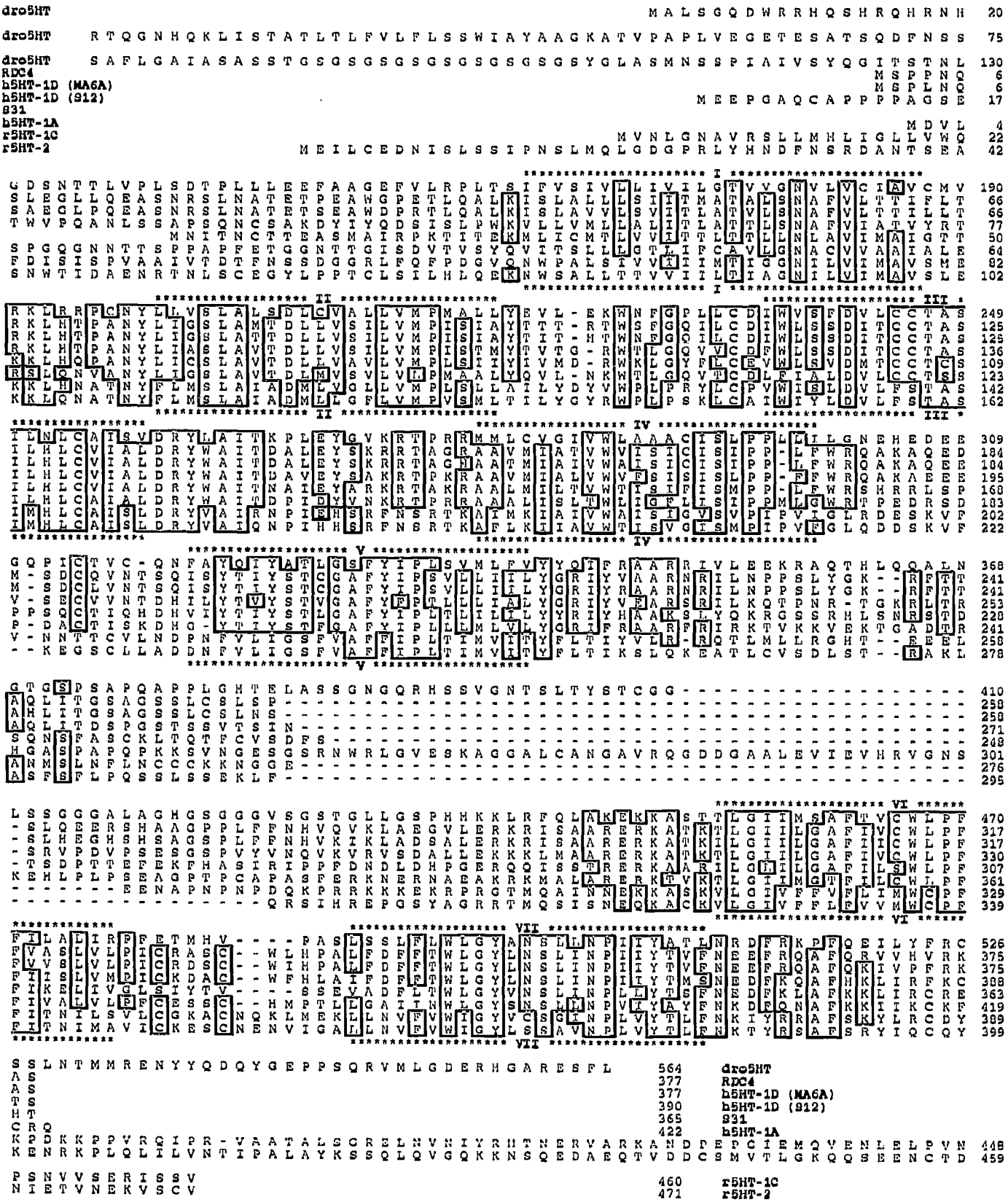
dro5HT
RDC4
h5HT-1D (MA6A)
h5HT-1D (912)
931
h5HT-1A
r5HT-1C
r5HT-2
I
II
III
IV
V
VI
VII
564 dro5HT
377 RDC4
377 h5HT-1D (MA6A)
390 h5HT-1D (912)
365 931
422 h5HT-1A
460 r5HT-1C
471 r5HT-2

Table I

Effects of various receptor ligands on adenylyl cyclase activity in L cells expressing the open reading frame encoded in clone S31

Additions to the assay	Adenylyl cyclase activity (pmol/min/mg protein)
PGE_1 10 μg/ml	30.6±0.3
NaF 10 mM	25.0±1.6
None	0.7±0.1
Forskolin 100 μM	35.9±1.3
+ 10 μM serotonin	23.5±0.6
+ 10 μM isoproterenol	33.3±1.0
+ 10 μM epinephrine	35.3±1.1
+ 10 μM histamine	32.6±0.9
+ 10 μM dopamine	36.7±0.6
+ 10 μM melatonin	33.3±0.7

Adenylyl cyclase activity was measured by determining the conversion of [α-^{32}P]ATP to [^{32}P]cAMP in total cell homogenates. LS31/27.9 cells were grown to close to confluency, split 1:5 into 15 cm dishes and used 48–60 h later. For this the medium was aspirated and the cells were washed twice with ice-cold BSS supplemented with 0.01% glucose, detached with the aid of a rubber policeman in the presence of BSS/0.01% glucose, and collected by centrifugation. After pelleting, the cells were resuspended in 1.5 ml/dish of ice-cold 27% w/w sucrose, 1 mM EDTA, 2 mM $MgCl_2$ and 20 mM Na-HEPES, pH 7.5, and homogenized in a 7-ml Dounce homogenizer (8 strokes with the tight pestle). The resulting homogenates were kept on ice for 15–30 min prior to assay. Aliquots of 10 μl (5 μg protein) were then tested for adenylyl cyclase activities as described previously [8] in the presence of the indicated compounds. Incubations were in triplicate for 20 min at 32°C. Results are means ± SD (n=3). Similar results were obtained for each of the compounds on at least three occasions.

Table II

Amino acid identity between homologous domains of cloned serotonin receptors

	RDC4	$h5HT_{1D}$ (MA6A)	$h5HT_{1D}$ (S12)	S31	$h5HT_{1A}$	$r5HT_{1C}$	$r5HT_2$
dro5HT	44.6	46.4	43.1	40.4	47.9	31.8	34.1
RDC4		90.3	74.3	56.6	51.9	35.4	34.3
$h5HT_{1D}$ (MA6A)			73.5	56.9	52.2	35.4	34.0
$h5HT_{1D}$ (S12)				59.2	53.0	34.3	34.3
S31					49.8	36.0	38.2
$h5HT_{1A}$						34.3	34.3
$r5HT_{1C}$							72.7

The table shows the amino-acid identity between the cloned serotonin receptors in and between the transmembrane regions (amino acid –1 from the beginning of the first TM segment through +11 after ending of TM5 and from –8 from the beginning of TM6 through +15 after ending of TM7). The compared sequences are: dro5HT, drosophila 5-HT receptor [11]; RDC4, the canine receptor-like sequence RDC4, showing highest homology to 5-HT receptors [9]; $h5HT_{1D}$ (MA6A), human 5-HT_{1D} receptor [7]; $h5HT_{1D}$ (S12), human 5-HT_{1D} receptor [8]; S31, the present novel human serotonin receptor; $h5HT_{1A}$, human 5-HT_{1A} receptor ('G-21' [2]); $r5HT_{1C}$, rat 5-HT_{1C} receptor [4]; $r5HT_2$, rat 5-HT_2 receptor [5,6].

terminal tail has the same short length as that of RDC4 and the two cloned human 5-HT_{1D} receptors (MA6A [7] and S12 [8]). A short intracellular tail seems to be a feature common to receptors coupled to inhibition of adenylyl cyclase. In this regard, the human 5-HT_{1A} receptor, which also inhibits adenylyl cyclase [23,24], has an intracellular tail that is only one amino acid longer (Fig. 4).

Table II shows that homologies amoung the serotonin receptors so far cloned. The percent identity was calculated over the two long stretches of highest similarity among the G protein-coupled receptors, i.e. transmembrane domains 1 through 5 and transmembrane domains 6 through 7, where only very few gaps had to be introduced to optimize the alignments (Fig. 4). As seen from Table II, receptor S31 shows greater similarity to the two cloned human 5-HT_{1D} receptors and RDC4 (59.2, 56.9, and 56.6% to 5-HT_{1D} (S12), 5-HT_{1D} (MA6A), and RDC4, respectively) than to any of the other receptors. On the other hand, the similarity of receptor S31 to any other receptor is lower than that between other functionally of pharmacologically related serotonin receptors (compare to 72.7% between rat 5-HT_{1C} and rat 5-HT_2, and 73.5% between the two cloned human 5-HT_{1D} receptors (MA6A and S12)).

3.3 *Demonstration that clone S31 encodes a functional serotonin receptor by expression in Ltk⁻ cells*

The identity of the S31 protein was then established by transfection into *Ltk⁻* cells, which have been used by us as model systems to test for receptor function [16–18]. A blunt-ended *Acc*I(–16)/*Bsp*HI(1140) fragment of pBS-S31.2, comprising 1156 nt of human genomic DNA containing the complete ORF, was subcloned into the expression vector pKNH (Fig. 1B) to give pK-S31, and transfected into *Ltk⁻* cells. Twenty-two G418-resistant colonies were picked, expanded, and tested for adenylyl cyclase activity in the absence and presence of serotonin. Of these, 7 cell clones showed between 15 and

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Fig. 4. Primary amino acid sequence comparison between cloned serotonin receptors. The represented sequences are: dro5HT, *Drosophila* 5-HT receptor [11]; RDC4, the canine receptor-like sequence RDC4, showing highest homology to 5-HT receptors [9]; h5HT-1D (MA6A), human 5-HT_{1D} receptor [7]; h5HT-1D (S12), human 5-HT_{1D} receptor [8]; S31, the present novel human serotonin receptor; h5HT-1A, human 5-HT_{1A} receptor ('G-21' [2]); r5HT-1C, rat 5-HT_{1C} receptor [4]; r5HT-2, rat 5-HT_2 receptor [5,6]. Gaps introduced to optimize the alignments are represented with dashes (–). The boxes indicate amino acids that are identical in at least five of the eight compared receptors. The seven putative transmembrane domains of the human S31 receptor are indicated with stars above and below the sequences, and have been derived by a combination of hydropathy analysis [25] and comparison with the suggested transmembrane domains of the other G protein-coupled receptors. The amino-acid sequence shown for the human 5-HT_{1A} receptor is that determined by us [8].

29% inhibition of adenylyl cyclase in response to serotonin. Several clonal cell lines were derived by limiting dilution from these initial cell clones. The inhibitory response to serotonin persisted. One cell line, LS31/27.9, was expanded and used for the experiments shown in Table I. As seen from the table, serotonin consistently gave a 30–35% inhibition of forskolin-stimulated adenylyl cyclase activity in homogenates from the clonal cell line LS31/27.9, expressing the putative receptor. No significant inhibition of forskolin-stimulated adenylyl cyclase activity was observed with any of the other drugs tested (isoproterenol, epinephrine, histamine, dopamine, and melatonin). Also, parental *Ltk*$^-$ cells were unresponsive to serotonin (not shown).

Taken together, these results strongly suggest that clone S31 encodes a functional G protein-coupled serotonin-receptor mediating inhibition of adenylyl cyclase. The relatively low homology of this novel serotonin-receptor to other cloned serotonin-receptors may indicate that it will also display a novel pharmacological profile.

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