Cloning of a melatonin-related receptor from human pituitary

Steven M. Reppert[a],*, David R. Weaver[a], Takashi Ebisawa[a],**, Cathy D. Mahle[b],
Lee F. Kolakowski Jr.[c]

[a]Laboratory of Developmental Chronobiology, Massachusetts General Hospital and Harvard Medical School, Jackson 1226, Boston, MA 02114, USA
[b]CNS Neuropharmacology, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT 06492, USA
[c]Department of Pharmacology, University of Texas Health Science Center in San Antonio, San Antonio, TX 78284-7764, USA

Abstract We have cloned an orphan G protein-coupled receptor from a human pituitary cDNA library using a probe generated by PCR. The cDNA, designated H9, encodes a protein of 613 amino acids that is 45% identical at the amino acid level to the recently cloned human Mel$_{1a}$ and Mel$_{1b}$ melatonin receptors. Structural analyses of the encoded protein and its gene, along with phylogenetic analysis, further show that H9 is closely related to the G protein-coupled melatonin receptor family. Unusual features of the protein encoded by H9 include a lack of N-linked glycosylation sites and a carboxyl tail >300 amino acids long. H9 transiently expressed in COS-1 cells did not bind [^{125}I]melatonin or [^{3}H]melatonin. H9 mRNA is expressed in hypothalamus and pituitary, suggesting that the encoded receptor and its natural ligand are involved in neuroendocrine function.

Key words: Melatonin; Human pituitary; G protein-coupled receptor

1. Introduction

Melatonin, the principal hormone of the pineal gland, influences the timing of mammalian circadian rhythms and regulates the reproductive alterations that occur in response to changes in day length in seasonally breeding mammals [1]. Melatonin elicits these responses through pharmacologically specific, guanine nucleotide binding protein (G protein)-coupled receptors.

Recently, a family of G protein-coupled melatonin receptors have been cloned from *Xenopus laevis*, chickens and mammals [2-5]. These recombinant receptors exhibit similar affinity and pharmacological characteristics to each other and to endogenous receptors, as defined with the melatonin agonist 2-[^{125}I]iodomelatonin ([^{125}I]Mel). Two mammalian melatonin receptor subtypes have been identified by molecular cloning studies. The mammalian Mel$_{1a}$ melatonin receptor is expressed in the hypothalamic suprachiasmatic nuclei (SCN) and hypophyseal pars tuberalis, presumed sites of the circadian and some of the reproductive actions of melatonin, respectively [3]. The mammalian Mel$_{1b}$ melatonin receptor is expressed in retina and brain and may mediate the reported effects of melatonin on retinal physiology in mammals [4]. A third receptor subtype, the Mel$_{1c}$ melatonin receptor, has been cloned from zebrafish, *Xenopus* and chickens, but not from mammals [5].

*Corresponding author. Fax: (1) (617) 726-1694.
E-mail: reppert@helix.mgh.harvard.edu

**Present address: Department of Psychiatry, Saitama Medical School, 38 Morohongo, Moroyama-cho, Iruma-gun, Saitama 350-04, Japan.

We now report the cloning and characterization of H9, a cDNA which encodes a newly described G protein-coupled receptor. Structurally, H9 is closely related to the cloned high-affinity melatonin receptor family, but expression studies show that it does not bind either [^{125}I]Mel or [^{3}H]melatonin. H9 mRNA is expressed in pituitary and hypothalamus. This pattern of expression suggests that the receptor encoded by H9 may have a role in human neuroendocrine function.

2. Materials and methods

2.1. PCR

For PCR with degenerate primers, genomic DNA was subjected to 30 cycles of amplification with 200 nM (final concentration) each of two oligonucleotide primers. Each reaction cycle consisted of incubations at 94°C for 45 s, 45°C for 2 min and 72°C for 2 min, with AmpliTaq DNA polymerase (Perkin-Elmer Cetus). For PCR with specific primers, genomic DNA or first-strand cDNA was subjected to 25–35 cycles of amplification using incubations at 94°C for 45 s, 60°C for 45 s and 72°C for 2 or 3 min. Amplified DNA was separated on an agarose gel and paper-eluted as previously described [3]. The DNA was then subcloned into pCRII using a TA cloning kit (Invitrogen), or digested with restriction enzymes and subcloned into pBluescriptII (Stratagene).

2.2. Library screening

Human pituitary and brain cDNA libraries in lambda gt10 and a human genomic library in EMBL-3 SP6/T7 (all from Clontech) were plated and transferred to Colony Plaque Screen filters (New England Nuclear). The filters were screened under conditions of high stringency, as previously described [3]. Lambda phage that hybridized to the probe were plaque-purified.

2.3. Rapid amplification of cDNA ends (RACE)

5'-RACE was performed using the 5'-AmpliFINDER RACE Kit (Clontech). First strand cDNA was generated by reverse transcription using primer 5'-GGGTAGCTTACAGCCAATACAGCCATA-3' based on sequence in the amino terminus of H9. The AmpliFINDER anchor was ligated to the cDNA ends using T4 RNA ligase. The cDNA was then amplified using an anchor primer (complementary to the ampliFINDER anchor) and primer 5'-ATGTTGCTCCTGGG-GATCGTTAAGA-3' based on sequence nested just upstream of the primer used for reverse transcription. The amplified cDNA was separated on an agarose gel. A specific band at ca. 180 bp was paper eluted, subcloned into pCRII, and sequenced.

2.4. Phylogenetic analysis

Melatonin receptors, H9 and the rat μ opioid receptor (outgroup) were aligned using CLUSTAL W [6]. The alignments were adjusted manually, and the regions corresponding to transmembrane segments 4–7, as defined by Baldwin [7], were utilized to generate a PAUP data matrix [8]. A branch and bound search was performed. The most parsimonious tree was found with a tree length of 765. Using PAUP, 1000 replicates of bootstrap analysis were performed. A neighbor-joining tree was calculated using CLUSTAL W and analyzed using the bootstrap procedure [6]. The neighbor-joining tree and the maximum parsimony trees do not differ, although the bootstrap values do differ.

2.5. Binding studies

COS-1 cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (50 µg/ml), in 5% CO_2 at 37 C.

For ligand binding studies, H9 in pcDNA3 was introduced into COS-1 cells using the DEAE-dextran method [9]. Three days after transfection, medium was removed, and crude membranes were prepared as described [4]. Binding assays were performed in duplicate in a final volume of 200 µl, at 37°C for 60 min or 0°C for 30 min. Nonspecific binding was defined using 1000-fold excess of unlabeled ligand. Protein measurements were performed by the method of Bradford [10].

2.6. Northern analysis

Poly(A)$^+$ RNA was obtained from human pituitary and hypothalamus as previously described. The RNA was separated by electrophoresis through a 1% agarose-formaldehyde gel, blotted onto Gene-Screen (New England Nuclear), and hybridized with random prime labeled probe. Hybridization occurred in 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 100 µg/ml salmon denatured salmon sperm at 42°C overnight. Final washing was for 1 h at 65°C in 2×SSC, 1% SDS. Blots were exposed at −80°C to X-ray film with an intensifying screen. Northern blots of human tissues (Human I and Human II) were also purchased from Clontech and hybridized and washed as recommended by the manufacturer.

2.7. In situ hybridization

^{35}S-labeled antisense cRNA probes were generated by digestion of pCRII containing a PCR-generated fragment of exon 2 with an appropriate restriction endonuclease, followed by in vitro transcription with T7 RNA polymerase (Ambion) in the presence of [^{35}S]α-thio-UTP (New England Nuclear, Boston MA; 1100–1200 Ci/mmol) as previously described [11]. As a control, sense cRNA probes were generated from the plasmid and processed along with the antisense probes. In each case, sense probes applied to sections produced a low, homogeneous level of background hybridization.

In situ hybridization was performed as previously described [11]. Film autoradiograms were generated by apposing slides to Kodak SB-5 X-ray film (Eastman Kodak, Rochester NY) for 18 days.

2.8. DNA sequencing

Nucleotide sequences were analyzed by the dideoxynucleotide chain termination method of Sanger et al. [12], using Sequenase (United States Biochemical). Sequencing template was double-stranded plasmid. Primers were synthetic oligonucleotides that were either vector specific or derived from sequence information.

2.9. Drugs

[^{125}I]Mel was purchased from New England Nuclear. All drugs used in binding studies were purchased from Sigma, Research Biochemicals or synthesized locally. All other chemicals were purchased from Sigma.

3. Results

3.1. Isolation of a melatonin-related receptor clone

To clone melatonin receptor subtypes, we used PCR amplification of human genomic DNA with degenerate oligonucleotide primers based on conserved amino acid residues in the third and sixth transmembrane domains of the cloned melatonin receptors. A novel cDNA fragment (364 bp) was found by sequence analysis to be 40% identical at the amino acid level with either the human Mel$_{1a}$ or Mel$_{1b}$ melatonin receptors. This PCR fragment was random prime labeled and used to probe human pituitary and brain cDNA libraries at high stringency. Positively hybridizing clones were found only with the pituitary library. Restriction endonuclease mapping and sequence analysis of several positive phage clones identified three overlapping clones that collectively contain an open reading frame encoding a protein of 613 AA (estimated mol. wt. of 66 923). The flanking sequence of the first methio-

nine in this reading frame displays a Kozak consensus sequence [13] for the initiation of translation. The three overlapping clones were digested with restriction endonucleases and then ligated together to produce clone H9 (Fig. 1).
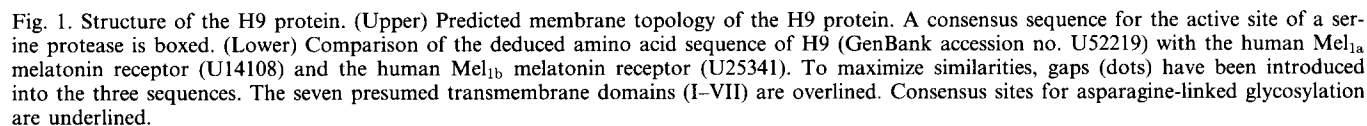
3.2. Receptor structure

Hydropathy analysis of the predicted amino acid sequence of H9 using the method of Kyte and Doolittle [14] revealed the presence of seven hydrophobic segments and a hydrophilic carboxyl tail (data not shown). H9 appears to be closely related to a newly described melatonin receptor group that is distinct from the other receptor groups (e.g. biogenic amine, neuropeptide, and photopigment receptors) that comprise the prototypic G protein-coupled receptor superfamily [2–5]. At the amino acid level, H9 shows highest identity with the recently cloned melatonin receptor family and is 45% identical overall to the human Mel$_{1a}$ and Mel$_{1b}$ receptors. The identity increases to 55% when only the transmembrane domains are compared. Unique features of the melatonin receptor group which are also found for H9 include an NRY sequence just downstream from the third transmembrane domain (rather than DRY) and a NAXXY sequence in transmembrane 7 (rather than NPXXY) (Fig. 1, lower). In addition, H9 and the human melatonin receptors all have a CYICHS sequence immediately downstream from NRY in the third cytoplasmic loop which is a consensus site for cytochrome c family heme binding [15]. The functional significance of this sequence is not yet known.

Distinguishing features of H9 include no consensus sites for N-linked glycosylation in either the amino terminus or extracellular loops (Fig. 1). Also, the carboxyl tail of H9 is over 300 amino acids long. The carboxyl tail is proline-rich (37 residues) and contains a consensus sequence for the catalytic site of a serine protease (boxed). The carboxyl tail has 41 serine and 18 threonine residues that may function as phosphorylation sites.

3.3. Gene structure

The major transcription start site for H9 was determined using 5'-RACE (see Section 2). Sequence analysis of 5'-RACE clones showed that they are all identical, with the cap site 138 bp upstream of the translation start codon. In the 5' untranslated region, the cDNA sequence matched the sequence or genomic clones, indicating that introns are not present in this region. Restriction endonuclease mapping and PCR analysis of genomic clones showed that the H9 gene is comprised of at least 2 exons, separated by an intron that is 3.0 kb in length (Fig. 2A). The intron has consensus 5'-GT-AG-3' sequence for splicing, and the splice sites occur at exactly the same location in the coding region as those described for both the human Mel$_{1a}$ and Mel$_{1b}$ receptor genes (Fig. 2B). Exon 1 encodes the entire 5' untranslated region and the coding region through the first cytoplasmic loop. Exon 2 encodes the rest of the coding region. It may also encode the 3' untranslated region, but this remains to be determined. Southern analysis of human genomic DNA digested with six different restriction endonucleases was performed using a PCR fragment of the second exon of H9 as a hybridization probe. Under high stringency conditions, we observed single-band patterns, suggesting that H9 is a single copy gene (data not shown).

Fig. 1. Structure of the H9 protein. (Upper) Predicted membrane topology of the H9 protein. A consensus sequence for the active site of a serine protease is boxed. (Lower) Comparison of the deduced amino acid sequence of H9 (GenBank accession no. U52219) with the human Mel$_{1a}$ melatonin receptor (U14108) and the human Mel$_{1b}$ melatonin receptor (U25341). To maximize similarities, gaps (dots) have been introduced into the three sequences. The seven presumed transmembrane domains (I–VII) are overlined. Consensus sites for asparagine-linked glycosylation are underlined.

3.4. Evolutionary relationship between H9 and melatonin receptors

We generated an alignment of the regions of H9 and melatonin receptors from transmembrane segments 4–7, with the rat μ opioid receptor serving as an outgroup. This alignment was used to calculate the most parsimonious phylogenetic tree using the program PAUP [8]. This tree showed that H9 is closely related to the three melatonin receptor subtypes (Fig. 3). A very similar tree was obtained using the neighbor-join method [16]. Bootstrap analyses of the tree and the data set showed that the assignment of H9 as a new member of the melatonin receptor cluster is strongly supported.

3.5. Ligand binding studies

To determine whether H9 encodes a melatonin receptor, binding and pharmacological properties were examined by transiently expressing H9 in COS-1 cells. H9 did not specifically bind 10 nM [^{125}I]Mel, [^{3}H]melatonin, [^{3}H]serotonin, [^{3}H]carboxamidotryptamine maleate, [^{3}H]N-acetylserotonin, [^{3}H]prazosin or [^{3}H]tryptamine at 37°C. It also did not specifically bind 0.2 nM [^{125}I]Mel at 0°C. Parallel experiments (at 37°C) showed that COS-1 cells transfected with either the human Mel$_{1a}$ or Mel$_{1b}$ receptor cDNAs bound [^{125}I]Mel with high affinity, as previously described [3,4].

3.6. Distribution of H9 mRNA

Northern blot analysis of RNA from human pituitary probed with a cDNA fragment from exon 2 revealed a major hybridizing transcript at 6.0 kb and a minor one at 9.5 kb (Fig. 4). A weak hybridization signal at 6.0 kb was also observed in hypothalamus. No hybridization signal was apparent in whole brain, spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes, heart, placental, lung, liver skeletal muscle kidney and pancreas (data not shown).

The anatomical pattern of expression of H9 mRNA was assessed by in situ hybridization (Fig. 4). Coronal sections (15 μm) from three subjects were examined at ca. 200 μm intervals from the optic chiasm through the tuberal region. A specific hybridization signal was apparent in the mediobasal hypothalamus of one subject, in a region including the ventromedial and arcuate nuclei of the hypothalamus. The region of the paraventricular nucleus of the hypothalamus also contained specific hybridization in this subject. Sections from the other two subjects did not include these regions. H9 was also expressed in the pituitary gland and infundibular stalk. Within the pituitary gland, expression was widespread and heterogeneous.

Near-adjacent sections from each subject were processed for in vitro autoradiography with [125I]Mel (40 pM; non-specific binding determined with 1 μM melatonin). In each subject, specific [125I]Mel binding was present in the SCN, but was not detected in the regions expressing H9 mRNA.

4. Discussion

H9 encodes a putative G protein-coupled receptor that is closely related to the recently defined melatonin receptor group. Structurally, there are remarkable similarities between H9 and the cloned melatonin receptors which distinguish them from other receptor groups within the prototypic G
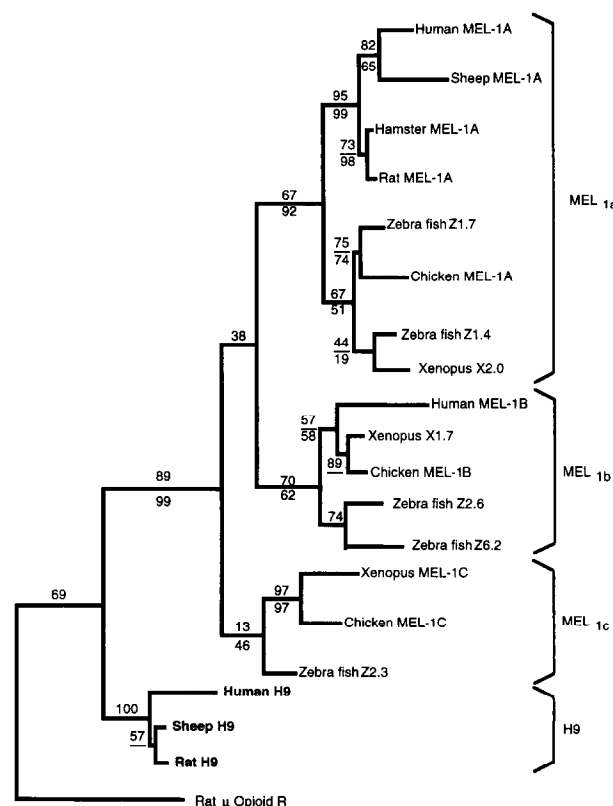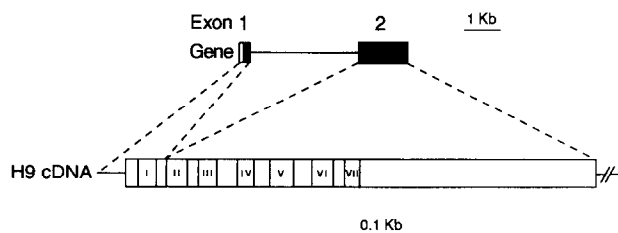

Fig. 3. Phylogenetic analysis of H9 and melatonin receptors. The regions comprising transmembrane segments 4–7 were used to calculate a tree using the maximum parsimony method. The numbers above and below the branches are the results of bootstrap analysis and are related to a confidence limit for the positions of the branches. The rat μ opioid receptor sequence provided an outgroup for the tree. GenBank accession numbers not previously published (see reference 5) are U52220 (Z6.2), U52221 (sheep H9), and U52218 (rat H9).

protein-coupled receptor superfamily. In addition, the gene structure of H9 is very similar to that of the Mel_{1a}, Mel_{1b} and Mel_{1c} melatonin receptors. Moreover, phylogenetic analysis confirms a close resemblance between H9 and the high-affinity melatonin receptors (Fig. 3), suggesting that they all evolved from the same ancestral gene.

H9 has some unusual structural features. First, it lacks N-linked glycosylation sites in the amino terminus and extracellular loops. Another G protein-coupled receptor, the α_{2B}-adrenergic receptor, has been reported which does not contain N-linked glycosylation sites [17-19]. This lack of glycosylation sites for the recombinant adrenergic receptors of rats, humans and mice is consistent with biochemical analysis of purified endogenous protein which does not have any associated oligosaccharide moieties [20]. Importantly, the endogenous and recombinant α_{2B}-adrenergic receptors exhibit high-affinity ligand binding characteristics. Thus, it seems unlikely that the lack of glycosylation sites has significant functional consequences or contributes to our inability to detect ligand binding for H9.

A second unusual feature of H9 is the length of its carboxyl tail. The tail length is longer than that reported for any other cloned prototypic G protein-coupled receptor (search of G protein-coupled receptor data base). Based on studies with other G protein-coupled receptors, the carboxyl tail may be
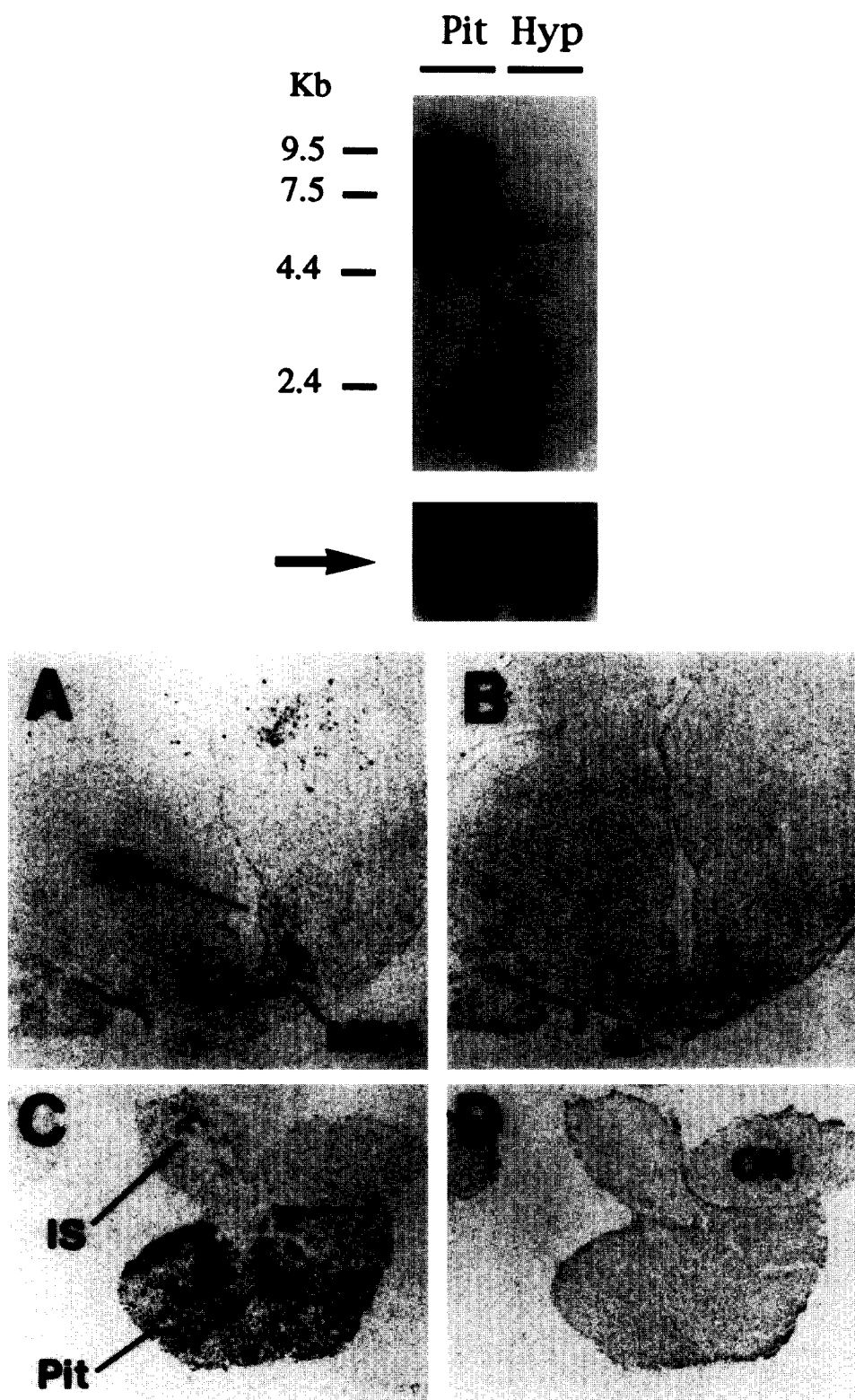

Fig. 2. Structure of the H9 gene. A: The upper portion depicts the receptor gene with exons illustrated as bars. Translated regions of the exons are black, while the 5' untranslated region is open. Below the gene, the receptor cDNA is shown with the coding region depicted as a wide bar; transmembrane domains I–VII are indicated. The thin lines depict the untranslated regions. Dashed lines between the gene and cDNA show the portions of the gene encoding the cDNA. B: Location and consensus sequence of exon-intron splice sites are shown.

Fig. 4. Distribution of H9 mRNA. (Top) Northern blot analysis. Each lane contained 3 µg of poly(A)$^+$ RNA. RNA size markers are indicated. Blot was exposed to X-ray film for 3 days. The lower portion of the blot depicts hybridization pattern using an actin probe to verify equal loading of lanes (arrow). (Bottom) In situ hybridization. (A,C) Hybridization signal is present in the mediobasal hypothalamus (MBH), infundibular stalk (IS), and pituitary gland (Pit) of sections hybridized with an antisense cRNA probe. (B,D) Non-specific hybridization of the sense control probe to adjacent sections at each level. ON, optic nerve; 3V, third ventricle. The width of each panel represents 1.4 mm actual size.

involved in receptor coupling to G proteins, tethering the receptor to the plasma membrane or phosphorylation events [21]. The identification of a consensus site for a serine protease

in the carboxyl tail of H9 raises the interesting possibility that the receptor tail has enzymatic function, which may depend on receptor activation and/or phosphorylation state. The tail

may also be a site of proteolysis with cleaved portions of the tail serving some intracellular function.

The natural ligand for the receptor encoded by H9 is likely a molecule related to melatonin. Our ligand binding studies strongly suggest that H9 does not bind melatonin, although we did not prove that the receptor protein is actually expressed and intercalated into the plasma membrane of COS-1 cells. Consistent with our ligand binding studies is the finding that H9 mRNA is expressed in structures that do not bind [^{125}I]Mel. We have also cloned cDNA fragments from rats and sheep that are species homologs of H9 (Fig. 3). In these animals, the expression pattern of H9 mRNA by in situ hybridization also does not match the [^{125}I]Mel binding pattern (unpublished). The expression of H9 mRNA in human hypothalamus and pituitary suggests that the receptor and its ligand are involved in the regulation of neuroendocrine function.

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