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The Ovine Melatonin-Related Receptor: Cloning and Preliminary Distribution and Binding Studies

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Abstract

A melatonin-related receptor was cloned from an ovine genomic library. The sequenced gene has a similar structure to that of the melatonin receptor gene family and consists of two exons separated by an intron of approximately 3 kb. Exon 1 and exon 2 of the ovine melatonin-related receptor encode a protein of 575 amino acids which is 73.8% homologous to the human melatonin-related receptor and shows 40.9% homology with the ovine Mel_{1a} melatonin receptor. COS-7 cells transiently expressing ovine melatonin-related receptors did not bind 2-[^{125}I]iodomelatonin or ^{3}H-melatonin. Reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization studies revealed expression of the ovine melatonin-related receptor in the hypothalamus, pituitary, retina and retinal pigment epithelium. Furthermore, expression of the ovine melatonin-related receptor is shown to be coincident with Mel_{1a} and 2-[^{125}I]iodomelatonin binding in the pituitary and serotonin *N*-acetyl transferase (arylalkylamine *N*-acetyl transferase, AANAT) expression in the retina. Expression patterns and similarity with the melatonin receptor gene family suggest a role for this novel G protein-coupled receptor in control and regulation of endocrine function and retinal physiology.

An orphan human melatonin-related receptor with 45% identity at the amino acid level to the family of G protein-coupled melatonin receptors was cloned recently by Reppert *et al.* (1). Melatonin and the G protein-coupled melatonin receptors have a well defined role in neuroendocrine regulation and control of seasonality and circadian rhythmicity with receptors localized in the hypothalamus and pituitary (2–5). Melatonin receptors have also been implicated in retinal physiology (6–10). Melatonin receptors bind the methoxyindole melatonin, which is synthesized at night in the pineal gland from serotonin via a well defined pathway: serotonin is *N*-acetylated by AANAT and then converted to melatonin by hydroxyindole-O-methyl transferase (HIOMT) (11). COS-1 cells transiently transfected with human melatonin-related receptors did not bind the melatonin agonist 2-[^{125}I]iodomelatonin or ^{3}H-melatonin (1). However, ligand binding studies using the human melatonin-related receptor did not establish whether the transfected cells were expressing the receptor protein. It was also reported that the expression pattern of a cloned ovine melatonin-related receptor cDNA fragment did not correspond to 2-[^{125}I]iodomelatonin binding in sheep tissue (1).

We report here the cloning of the complete coding sequence of an ovine melatonin-related receptor and identify sites of its mRNA expression in the hypothalamus, pituitary, retina and retinal pigment epithelium (RPE). Ligand binding studies were performed on transfected COS-7 cells using FLAG epitope tagged receptors to confirm that the expressed ovine melatonin-related receptors do not exhibit high or low affinity binding for the melatonin analogue 2-[^{125}I]iodomelatonin or ^{3}H-melatonin. Further experiments were performed to analyse expression of the ovine melatonin-related receptor in tissues showing 2-[^{125}I]iodomelatonin binding and coincident expression of AANAT, which is known to be involved in synthesis of the melatonin receptor ligand melatonin.

Results

Cloning of the ovine melatonin-related receptor gene

PCR of ovine genomic DNA using degenerate primers amplified a DNA fragment encoding transmembrane segments three to seven of the ovine melatonin-related receptor. The ovine melatonin-related receptor cDNA was used to probe

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an ovine genomic library. Positive hybridizing clones were plaque purified and used for preparation of DNA. Restriction enzyme analysis of the prepared (DNA yielded a 4 kb *Hind*III fragment consisting of the complete coding sequence of exon 2 and an intron sequence of approximately 2 kb (Fig. 1).

A repeat library screen using an intron specific oligo probe hybridizing to a region situated at the 5′ end of the sequenced 4 kb *Hind*III fragment (Fig. 1) yielded an overlapping clone. DNA prepared from the clone hybridizing to the intron specific oligo was cleaved downstream of the intron specific sequence yielding a *Sal*I/*Xba*I fragment (approximately 6 kb in size) which was subcloned in pBluescript SK (Stratagene, La Jolla, USA) and sequenced. This overlapping fragment encoded an amino acid sequence homologous to exon 1 of the human melatonin-related receptor. Figure 2 shows the sequence and predicted polypeptide of the ovine melatonin-related receptor gene. The position and splicing consensus sequence 5′ GT-AG 3′ of the intron are shown in Fig. 2. The intron was determined to be approximately 3 kb from restriction analysis of the isolated genomic clones. The position of the intron is identical to that of the sequenced melatonin receptors (5, 10) and the human melatonin-related receptor (1). Exon 1 and exon 2 were spliced using PCR to construct an overlapping cDNA that was cleaved with restriction enzymes and ligated to exon 2 and cloned in pcDNA3 (Invitrogen). The full length construct was verified by DNA sequencing.

Amino acid sequence comparison

The ovine melatonin-related receptor gene encodes an amino acid sequence of 575 amino acids with an estimated molecular weight of 62 817. A BLAST sequence similarity search of the polypeptide predicted by the ovine exon 1 and exon 2 against GenBank data revealed highest homology with the human melatonin-related receptor (U52219, 73.8% overall, increasing to 94.8% from transmembrane segments three to seven) and the sheep (U52221, 94.9%) and rat (U52218, 84%) melatonin-related receptor cDNA fragments. The sheep melatonin-related receptor has 40.9% similarity with the ovine $Mel_{1a\alpha}$ melatonin receptor (U14109), increasing to 46.9% between transmembrane segments three to seven. An alignment showing comparisons with related sequences is shown in Fig. 3. A Kyte and Doolittle (12) hydropathy plot supports the presence of seven hydrophobic regions corresponding to the seven transmembrane domains indicated in Fig. 3. The ovine melatonin-related receptor fragment generated by PCR (1) reveals some sequence variation with the genomic clone isolated. The following residues encoded by the genomic clone, arginine (position 138) and leucine (position 158) are replaced by a leucine and a methionine, respectively, and alanine and arginine (positions 218–219) are deleted from the ovine melatonin-related receptor PCR generated fragment. The sequence variation may be a consequence of PCR errors generated by the DNA polymerase used for PCR or alternatively may represent allelic variation. The ovine melatonin-related receptor has several similarities with the human melatonin-related receptor having no *N*-linked glycosylation in either the amino terminus or extracellular loops and a long carboxyl tail of 280 amino acids. Similar to the human melatonin-related receptor the carboxyl tail is rich in proline (25 residues), serine (37 residues) and threonine (24 residues). The amino acid sequence was scanned against PROSITE (ExPASy WWW molecular biology server, Geneva Hospital and the University of Geneva, Switzerland) revealing several putative phosphorylation sites (Fig. 2).

Expression and ligand binding studies of the ovine melatonin-related receptor in COS-7

Expression of the ovine melatonin-related receptor was confirmed by immunofluorescence using an anti-FLAG M5 monoclonal antibody to the DYKDDDDK epitope at the *N*-terminus of the receptor (Fig. 4). The epitope tagged construct of the ovine melatonin-related receptor shows a similar distribution to that of the DYKDDDDK epitope tagged ovine $Mel_{1a\beta}$ receptor (13) (Fig. 4) which binds 2-[^{125}I]iodomelatonin. The epitope tagged receptors are

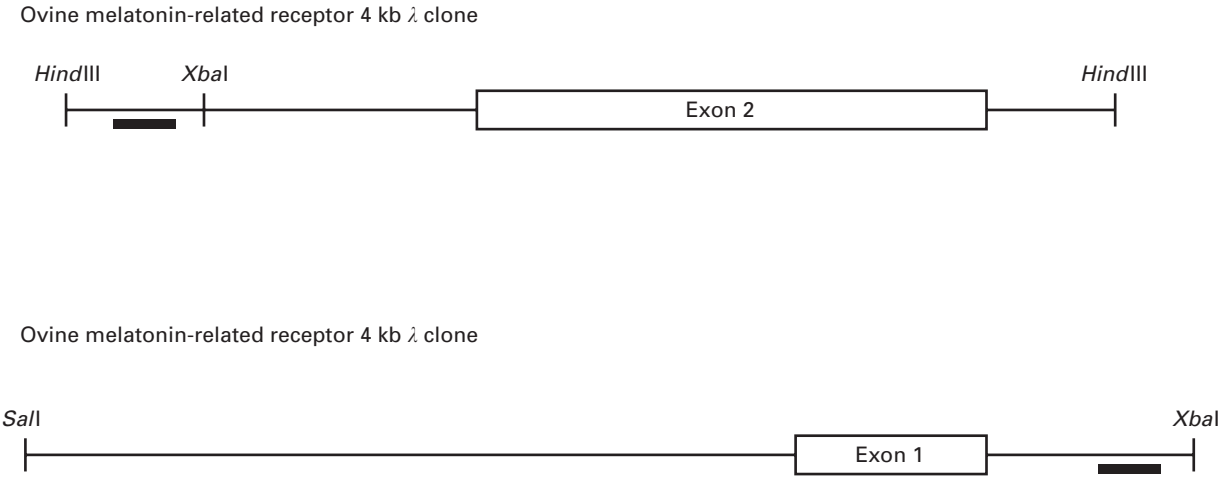

FIG. 1. Schematic diagram representing the subcloned overlapping fragments encoding the ovine melatonin-related receptor. The fragments were cleaved from λDNA clones isolated from a screen of the ovine genomic library. The intron specific oligo probe used to identify the genomic fragment encoding exon 1 is denoted by the bar.

distributed throughout the cell and are not compartmentalised within cellular organelles. Fluorescence was not observed when primary antibody was omitted, nor when cells were transfected with ovine melatonin-related receptor constructs without the epitope or mock transfected COS-7.

Examination of COS-7 transiently expressing the melatonin-related receptor for 2-[^{125}I]iodomelatonin binding revealed no high affinity binding at 100 pM. Low affinity binding of 2-[^{125}I]iodomelatonin and ^{3}H-melatonin at 10 nM also proved negative. In contrast the ovine $Mel_{1a\beta}$ [as described previously by Barrett *et al* (13)] and the epitope tagged $Mel_{1a\beta}$ showed specific binding to 2-[^{125}I]iodomelatonin and ^{3}H-melatonin (Fig. 5). This suggests that the FLAG epitope does not interfere with ligand binding of the receptors.

Localization of ovine melatonin-related receptor mRNA expression in tissues

Tissue specific melatonin-related receptor gene expression was studied using RT-PCR and *in situ* hybridization. RT-PCR performed on mRNAs from sheep tissues revealed expression of melatonin-related receptor mRNA in the pars tuberalis, pars distalis, retina and RPE. to determine the distribution of the melatonin-related receptor (Fig. 6). DNase treatment of the mRNA samples prior to RT-PCR did not eliminate amplification of the hybridizing products. No hybridizing products were obtained when processed without the addition of mRNA or RNase treatment prior to RT-PCR. The hybridizing RT-PCR products were subcloned and sequenced to confirm their identity as melatonin-related receptor sequences.

In situ hybridization was performed to localize melatonin-related receptor expression in sheep brain, pituitary, retina and RPE (Figs 7–9). Initial studies examined a series of coronal sections from sheep brain, with cortex removed, at 1 mm intervals from the preoptic area to beyond the hypothalamus. Discrete sites of mRNA expression were observed in a punctate pattern over a region of the hypothalamus. *In situ* hybridization was then performed on serial sections through the hypothalamus to identify the hypothalamic nuclei involved. Finally, sagittal sections were used to show the extent of the ovine melatonin-related receptor mRNA expressing tissues in the brain and pars tuberalis (Fig. 7). Near-adjacent sections were used to localize CRF expression to delineate the paraventricular nucleus (Fig. 7). Superimposed digital images of *in situ* hybridization to the ovine melatonin-related riboprobe (Fig. 7A) in the ovine brain and the CRF oligo probe (Fig. 7C) revealed no overlap. The CRF localizes to and delineates the paraventricular nucleus (14). The ovine melatonin-related receptor is not expressed in the PVN but is expressed in the dorsal medial hypothalamus situated below the PVN (Fig. 7). Near-adjacent sections were used to show 2-[^{125}I]iodomelatonin binding (Fig. 7) in the pars tuberalis. 2-[^{125}I]iodomelatonin binding was not observed over the dorsal medial hypothalamus.

Further analysis of expression in the pituitary gland by *in situ* hybridization detected widespread expression of melatonin-related receptor mRNA throughout the pituitary and coincident expression of Mel_{1a} and 2-[^{125}I]iodomelatonin binding in tissues of the pars tuberalis (Fig. 8). The melatonin-related receptor was also localized to the retina (Fig. 9). AANAT was also expressed in the retina (Fig. 9). Determination of specific hybridization of the melatonin-related receptor antisense riboprobe to the RPE was not possible due to observed nonspecific hybridization of the sense riboprobe (Fig. 9).

Discussion

We have cloned an ovine melatonin-related receptor, which on the basis of sequence comparisons is most likely a homologue of the recently cloned human melatonin-related receptor. The ovine melatonin-related receptor is a member of the melatonin receptor family as indicated by unique sequence motifs including an NRY (as opposed to DRY) in intracellular loop 2 and an NAXXY motif in transmembrane domain 7 (as opposed to NPXXY) (Fig. 3). Both the human and sheep melatonin-related receptors are characterized by a long carboxyl tail. The carboxyl tail of G protein-coupled receptors is thought to play a role in G protein coupling, desensitization and down regulation (15). Hence, the putative kinase sites may be significant (see Fig. 2). The ovine melatonin-related receptor unlike the human melatonin-related receptor does not have a consensus site for a serine protease in the carboxyl tail (1). Hence, the possibility of an enzymatic function serving to produce a cleaved receptor would not be conserved in the ovine melatonin-related receptor homologue.

Reppert *et al.* (1) reported localization of the human melatonin-related receptor in various nuclei of the hypothalamus in one subject studied, but not in others. Widespread and heterogeneous expression of the human melatonin-related receptor was also observed in the pituitary gland and infundibular stalk. This pattern of expression has some similarities with that of the ovine melatonin-related receptor. We have also noted widespread expression of the ovine melatonin-related receptor in the pituitary gland (Figs 7 and 8). Expression was also observed in the sheep hypothalamus (Fig. 7), but was observed consistently in discrete regions of the four sheep hypothalami examined. CRF localization to the PVN clearly shows that the melatonin-related receptor is not expressed in this nucleus but in an adjacent area, in contrast to that of the human melatonin-related receptor (1). Similarly, unlike the human melatonin-related receptor there is a close association of 2-[^{125}I]iodomelatonin binding with regions expressing the ovine melatonin-related receptor. This also contrasts to unpublished observations reported by Reppert *et al.* (1), where melatonin-related receptor expression did not coincide with 2-[^{125}I]iodomelatonin binding patterns in sheep. We observed consistently ovine melatonin-related receptor expression and 2-[^{125}I]iodomelatonin binding in pars tuberalis (Figs 7 and 8). Expression in tissues of the pituitary was confirmed by RT-PCR. RT-PCR also revealed expression of the ovine melatonin-related receptor in the retina and RPE. *In situ* hybridization confirmed localization of ovine melatonin-related receptor transcripts in retinal tissue. However, problems of nonspecific hybridization to the RPE prevented confirmation of expression in this tissue (Fig. 9). Melatonin binding sites (7) and melatonin receptors (10) have also been reported in the retina of several

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ATGGGACGCACCCTGGCGGTCCCTACCCCATACGGCTGTATCGGCTGCAAACTGCCACAA
 M  G  R  T  L  A  V  P  T  P  Y  G  C  I  G  C  K  L  P  Q

CCGGACTACCCACCGGCTCTAATCGTCTTTATGTTCTGTGCAATGGTTATCACCATCGTC
 P  D  Y  P  P  A  L  I  V  F  M  F  C  A  M  V  I  T  I  V

GTAGACCTGATCGGCAACTCTATGGTCATTTTGGCTGTGTCGAAGAACAAGAAGCTCCGA
 V  D  L  I  G  N  S  M  V  I  L  A  V  S  K  N  K  K  L  R

AATTCTGgt.................intron splice site.............
 N  S

.....agGCAACGTCTTCGTGGTTAGCCTCTCTGTGGCTGATATGCTGGTGGCCATCTAC
      G  N  V  F  V  V  S  L  S  V  A  D  M  L  V  A  I  Y

CCCTATCCTCTGATGCTACATGCCATGGCCATCGGCGGCTGGGATCTCAGCAAGTTACAG
 P  Y  P  L  M  L  H  A  M  A  I  G  G  W  D  L  S  K  L  Q

TGCCAGATGGTGGGGTTCATCACAGGCCTGAGTGTGGTCGGTTCTATCTTCAACATCATG
 C  Q  M  V  G  F  I  T  G  L  S  V  V  G  S  I  F  N  I  M

GCCATCGCCATCAACCGTTACTGCTACATCTGCCACAGCCTCCAGTATGAGCGCATCTTT
 A  I  A  I  N  R  Y  C  Y  I  C  H  S  L  Q  Y  E  R  I  F

AGTGTGCGCAATACCTGCATTTATCTGGCTGTCACCTGGATCATGACCGTTCTGGCTGTC
 S  V  R  N  T  C  I  Y  L  A  V  T  W  I  M  T  V  L  A  V

CTACCCAACATGTACATTGGCACCATCGAGTATGATCCTCGCACCTACACCTGCATCTTT
 L  P  N  M  Y  I  G  T  I  E  Y  D  P  R  T  Y  T  C  I  F

AACTATGTGAACAACCCTGCCTTTGCTGTGACCATCGTCTGCATCCACTTTGTCCTTCCT
 N  Y  V  N  N  P  A  F  A  V  T  I  V  C  I  H  F  V  L  P

CTGCTCATAGTGGGTTTCTGCTACGTGAAGATCTGGACCAAAGTGCTGGCAGCCCGTGAC
 L  L  I  V  G  F  C  Y  V  K  I  W  T  K  V  L  A  A  R  D

CCTGCTGGACAGAACCCGGACAACCAGCTTGCTGAGGTTCGAAATTTTCTAACCATGTTT
 P  A  G  Q  N  P  D  N  Q  L  A  E  V  R  N  F  L  T  M  F

GTGATCTTCCTCCTCTTTGCAGTGTGCTGGTGCCCTATCAATGCGCTCACTGTTCTGGTG
 V  I  F  L  L  F  A  V  C  W  C  P  I  N  A  L  T  V  L  V

GCTGTCAATCCGAAGGAGATGGCAGGCAAGATCCCCAACTGGGTTTATCTTGCAGCCTAC
 A  V  N  P  K  E  M  A  G  K  I  P  N  W  V  Y  L  A  A  Y

TTTATAGCCTACTTCAACAGCTGCCTCAACGCGGTGATATATGGTGTCCTCAATGAGAAT
 F  I  A  Y  F  N  S  C  L  N  A  V  I  Y  G  V  L  N  E  N

TTCCGAAGAGAATACTGGACCATCTTCCATGCGATGCGGCATCCTGTCCTGTTCCTCTCT
 F  R  R  E  Y  W  T  I  F  H  A  M  R  H  P  V  L  F  L  S

GGCCTCCTCACTGATGTCCGTGAGATGCAGGAGGCCCAAGCCCACACCCATGCCCGTGCC
 G  L  L  T  D  V  R  E  M  Q  E  A  Q  A  H  T  H  A  R  A

CGTGCCCGCACACAAGCCCATGAACAAGACCATGCCCATGCCTGTCCTGCTGTGGAGGAA
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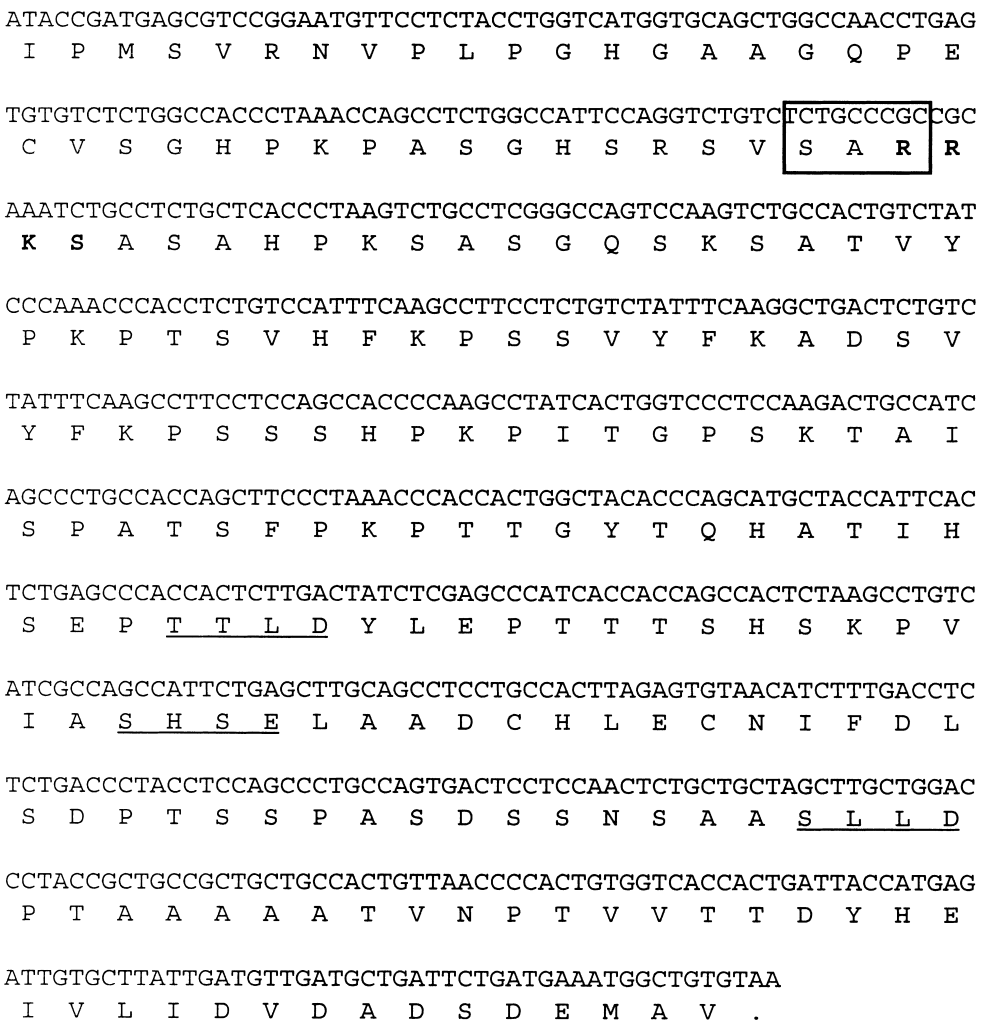

FIG. 2. (*Continued*).

mammalian species. Northern blotting experiments performed using 1 μg of mRNA from the tissues used in the RT-PCR failed to detect ovine melatonin-related receptor transcripts indicating low levels of expression in the tissues showing positive hybridizing RT-PCR products.

The similarity of the ovine melatonin-related receptor with the melatonin receptor family would suggest a natural ligand similar to melatonin. However, 2-[^{125}I]iodomelatonin high affinity (100 pM) and low affinity (10 nM) binding were not observed by COS-7 cells expressing the ovine melatonin-related receptor. Although 2-[^{125}I]iodomelatonin binding was observed in tissues of the pars tuberalis expressing the ovine melatonin-related receptor, this is attributed to the well characterized expression of the Mel_{1a} receptor in pars tuberalis (13, 16). This is supported by the absence of 2-[^{125}I]iodomelatonin binding in the pars distalis and the region of the hypothalamus expressing the ovine melatonin-related receptor. This does not preclude the possibility that the natural ligand for the ovine melatonin-related receptor is similar to melatonin and that it may be synthesized via a similar but divergent biosynthetic pathway. It is notable therefore that the mRNA encoding the enzyme AANAT is expressed in the pituitary (17) and retina (Fig. 9). There is evidence of melatonin synthesis in retinal and RPE tissues (8, 9, 18). However, it has been noted that HIOMT activity in the retina is either undetectable or very low in chicken, rat, cow and human in comparison to that of AANAT (19). Therefore, AANAT in the retina could play a role in melatonin synthesis or alternatively be involved in synthesis of a putative melatonin-related receptor ligand. To our knowledge, there are no reports of HIOMT activity in the pituitary. Conserved valine 208 and histidine 211 residues in transmembrane domain 5 of the melatonin receptor have recently been implicated in binding of the methoxy group of melatonin (20). The conservation of these residues in the melatonin-related receptor may be significant in identifying the native ligand. Further work will be necessary to determine the possibility of

FIG. 2. Ovine melatonin-related receptor gene sequence and predicted polypeptide. Consensus sequences for cAMP and cGMP-dependent protein kinase phosphorylation (bold); protein kinase C phosphorylation (boxed); casein kinase II phosphorylation (underlined) are marked.

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Ovine MRR      MGRTL---AVPTPYGCIGCKLPQPDYPPA-----------
Human MRR      MGPTL---AVPTPYGCIGCKLPQPEYPPA-----------
Ovine Mel1a    MAGRLWGSPGGTPKGNGSSALLNVSQAAPGAGDGVRPRPS

                                TMS I
                    ____________________________________
Ovine MRR      -LIVFMFCAMVITIVVDLIGNSMVILAVSKNKKLRNSGNV
Human MRR      -LIIFMFCAMVITIVVDLIGNSMVILAVTKNKKLRNSGNI
Ovine Mel1a    WLAATLASILIFTIVVDIVGNLLVVLSVYRNKKLRNAGNV

                               TMS II
                    ____________________________________
Ovine MRR      FVVSLSVADMLVAIYPYPLMLHAMAIGGWDLSKLQCQMVG
Human MRR      FVVSLSVADMLVAIYPYPLMLHAMSIGGWDLSQLQCQMVG
Ovine Mel1a    FVVSLAVADLLVAVYPYPLALASIVNNGWSLSSLHCQLSG

                              TMS III
                    ____________________
Ovine MRR      FITGLSVVGSIFNIMAIAINRYCYICHSLQYERIFSVRNT
Human MRR      FITGLSVVGSIFNIVAIAINRYCYICHSLQYERIFSVRNT
Ovine Mel1a    FLMGLSVIGSVFSITGIAINRYCCICHSLRYGKLYSGTNS

                               TMS IV
                    ____________________________________
Ovine MRR      CIYLAVTWIMTVLAVLPNMYIGTIEYDPRTYTCIFNYVNN
Human MRR      CIYLVITWIMTVLAVLPNMYIGTIEYDPRTYTCIFNYLNN
Ovine Mel1a    LCYVFLIWTLTLVAIVPNLCVGTLQYDPRIYSCTFTQSVS

                                TMS V
                    ____________________________________
Ovine MRR      PAFAVTIVCIHFVLPLLIVGFCYVKIWTKVLAAR--DPAG
Human MRR      PVFTVTIVCIHFVLPLLIVGFCYVRIWTKVLAAR--DPAG
Ovine Mel1a    SAYTIAVVVFHFIVPMLVVVFCYLRIWALVLQVRWKVKPD

                                                TMS VI
                                    ____________________
Ovine MRR      QNPDNQLAEVRNFLTMFVIFLLFAVCWCPINALTVLVAVN
Human MRR      QNPDNQLAEVRNFLTMFVIFLLFAVCWCPINVLTVLVAVS
Ovine Mel1a    NKPKLKPQDFRNFVTMFVVFVLFAICWAPLNFIGLVVASD

                                        TMS VII
                                ____________________
Ovine MRR      PKEMAGKIPNWVYLAAYFIAYFNSCLNAVIYGVLNENFRR
Human MRR      PKEMAGKIPNWLYLAAYFIAYFNSCLNAVIYGLLNENFRR
Ovine Mel1a    PASMAPRIPEWLFVASYYMAYFNSCLNAIIYGLLNQNFRQ

Ovine MRR      EYWTIFHAMRHPVLFLSGLLTDVREMQEAQAHTHARARAR
Human MRR      EYWTIFHAMRHPIIFFPGLISDIREMQEARTLARARAAHA
Ovine Mel1a    EYRKIIVSLCTTKMFF------------------------

Ovine MRR      TQAHEQDHAHACPAVEEIPMSVRNVPLPGHGAAGQPECVS
Human MRR      DQATEQDRAHACPAVEETPMNVRNVPLPGDAAAGHPDRAS
Ovine Mel1a    ----------------------------------------
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Ovine MRR     GHPKPAS-----------------GHSRSVSARRKSASA
Human MRR     GHPKPHSTSSSAYRKSASTHHKSVFSHSKAASGHLKPVSG
Ovine Mel1a   ----------------------------------------

Ovine MRR     HPKSASGQSKSATVYPKPTSVHFKPSSVYFKAD-------
Human MRR     HSKPASGHPKSATVYPKPASVHFKGDSVHFKGDSVHFKPD
Ovine Mel1a   ----------------------------------------

Ovine MRR     SVYFKPSSSHPKPITGP-------SKTAISPATSFPKPTT
Human MRR     SVHFKPASSNPKPITGHHVSAGSHSKSAFSAATSHPKP--
Ovine Mel1a   ----------------------------------------

Ovine MRR     GYTQHATIHSEPTTLDYLEPTTTSHSKPVIASHSELAAD-
Human MRR     --IKPATSHAEPTTADYPKPATTSHPKPAAADNPELSASH
Ovine Mel1a   ----------------------------------------

Ovine MRR     C-------HLECNIFDLSDPTSSPASDSSNSAASLLDPTA
Human MRR     CPEIPAIAHPVSDDSDLPESASSPAAGPTKPAASQLESDT
Ovine Mel1a   ----PLIAN-------------------------------

Ovine MRR     AAAATVNPTVVTT---DYHEIVLIDVDADSDEMAV
Human MRR     IADLP-DPTVVTTSTNDYHDVVVVDVEDDPDEMAV
Ovine Mel1a   ------------------HNLIKVD
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FIG. 3. (*Continued*).

FIG. 4. Immunocytochemical localization of ovine melatonin-related receptor. (A) COS-7 cells transfected with DYKDDDDK epitope tagged ovine melatonin-related receptor construct. (B) COS-7 cells transfected with wild type ovine melatonin-related receptor. (C) COS-7 cells transfected with DYKDDDDK epitope tagged ovine $Mel_{1a\beta}$ receptor construct. Bar = 50 μm.

autocrine or paracrine synthesis of a ligand for the ovine melatonin-related receptor in the pituitary or retina. A putative role for the ovine melatonin-related receptor in control and regulation of endocrine function and retinal physiology is suggested by its tissue distribution. Further corroboration of such a role awaits identification of its natural ligand and investigation of the intracellular signalling pathways mediated by this orphan receptor.

FIG. 3. Alignment of the ovine genomic melatonin-related receptor (MRR) sequence with the human melatonin-related receptor (MRR) cDNA from adult human pituitary (U52219) and the ovine Mel_{1a} melatonin receptor (U14109). The transmembrane domains are overlined. The conserved motifs indicative of the melatonin receptor family are underlined.

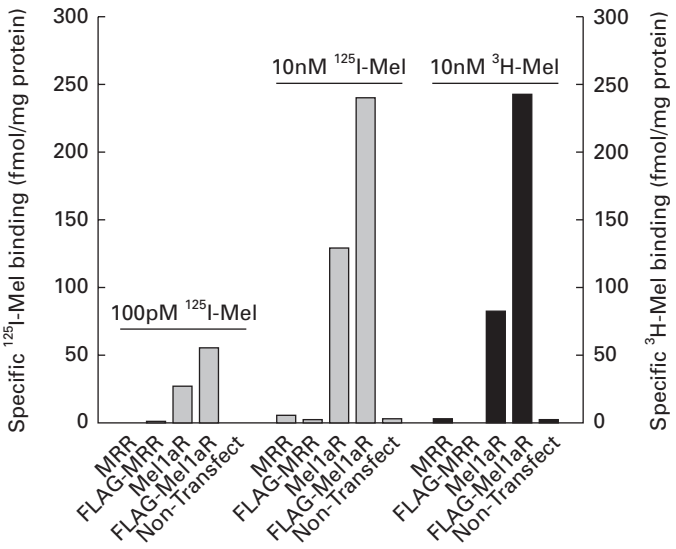

FIG. 5. 2-[^{125}I]Iodomelatonin binding analysis of the ovine melatonin-related receptor (MRR) and the $Mel_{1a\beta}$ receptor (Mel1aR) in their native and FLAG tagged forms transiently expressed in COS-7 cells. These are compared with nontransfected COS7 cells. The graph represents specific binding data from one typical representative experiment in the presence of 100 pM and 10 nM 2-[^{125}I]iodomelatonin (grey) and 10 nM ^{3}H-melatonin (black).

Materials and methods

Isolation of the ovine melatonin-related receptor gene

An ovine genomic library was constructed in the vector λEMBL3 (21). The library was plated ($5–10 \times 10^3$ plaques per 90 mm diameter Petri dish) and transferred onto GeneScreen nylon filters (NEN Dupont Ltd, Stevenage, Herts, UK). The filters were hybridized at 65 °C with a [^{32}P]dCTP (Amersham International, UK) random prime labelled PCR generated fragment consisting of transmembrane segments three to seven of the ovine melatonin-related receptor. The PCR generated fragment was obtained by performing PCR on an ovine genomic DNA template with degenerate oligo primers based on conserved amino acid residues in the third and seventh transmembrane domains of the cloned melatonin receptor (22). Reactions were performed in the presence of 100 pmol primer, 2.5 U of AmpliTaq, 200 μM dNTPs and 1.5 mM $MgCl_2$ $6H_2O$. The PCR reaction cycles consisted of 94 °C for 1 min, 45 °C for 1 min 30 s and 72 °C for 1 min 30 s for 35 cycles. The amplification products were subcloned using a pGEM-T vector system (Promega, Southampton, UK) and DNA was prepared for sequencing to confirm the identity of the PCR products.

Filters were washed to $0.2 \times$ SSC/0.1% SDS at 60 °C. Positive hybridizing clones were plaque purified and used for preparation of DNA using a Wizard Lambda DNA purification kit (Promega). Restriction fragments from the positive hybridizing (DNA clones were subcloned in pBluescript SK (Stratagene) and sequenced. Overlapping clones were obtained by re-screening the ovine genomic library with an intron specific oligo based on sequence information from the subcloned genomic fragments isolated above.

Construction of epitope tagged receptors

Both the ovine melatonin-related receptor and $Mel_{1a\beta}$ (13) were tagged with a FLAG epitope at the *N*-terminal. PCR was used on templates consisting of the ovine melatonin-related receptor cloned into pcDNA3 (Invitrogen) and ovine $Mel_{1a\beta}$ in pBK-CMV (Sratagene) (13) to insert a DNA sequence encoding a DYKDDDDK motif directly after the ATG methionine start codon. The reconstructed clones were sequenced to confirm correct insertion of the FLAG epitope.

Transfection of COS-7 cells

COS-7 cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum and 1% (v/v) antibiotic/antimycotic solution in 5% CO_2 at 37 °C. Transient transfections of COS-7 were performed on cells using Promega's Profection DEAE-dextran method. Cells were trypsinised 24 h later and seeded at a density of 1.25×10^5 per well in two well chambered slides (GibcoBRL Life Technologies Ltd, Paisley, UK) for immunocytochemistry and the remainder plated on 140 mm plates for use in ligand binding studies.

Immunocytochemistry of transfected COS-7 cells

Cells were transfected with DYKDDDDK epitope tagged receptors for immunocytochemistry to confirm expression of the melatonin-related receptor in transfected cells used for ligand binding. The transfected cells were fixed in the two well chambered slides with 4% paraformaldehyde/PBS (pH 7.3–7.5) for 20 min 48 h after transfection. The cells were incubated 3×5 min in PBS/1% normal horse serum/0.1% saponin prior to incubation overnight at 4 °C with anti-FLAG M5 monoclonal antibody (Kodak Scientific Imaging, Cambridge, UK) (0.5 μg/ml) in PBS. The cells were then incubated for 2 h at room temperature with horse-antimouse fluorescein conjugated secondary antibody (Vector Laboratories, Peterborough, UK) diluted in buffer (1 : 200) and viewed using fluorescence microscopy. Controls consisted of cells transfected with unflagged receptors, mock transfected cells, and treatment with and without primary antibody.

Ligand binding studies

Transfected cells were harvested 72 h after transfection by scraping gently off the plate, spinning down at 3000 **g** for 5 min and then freezing at −70 °C until required. The cells were thawed and washed twice in binding buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA). Aliquots of approximately 1×10^6 cells were incubated at 37 °C for 1 h with 100 pM 2-[^{125}I]iodomelatonin in 200 μl of binding buffer in the presence or absence of 10^{-6} M melatonin for detection of high affinity binding sites. Low affinity binding was determined using 1 nM 2-[^{125}I]iodomelatonin with cold iodomelatonin tracer to give a total concentration of 10 nM or 10 nM ^{3}H-melatonin in the presence or absence of 10^{-6} M melatonin. The binding assays were terminated as described previously (23). Specific counts for 2-[^{125}I]iodomelatonin were determined using a Packard Cobra γ–counter and specific counts for ^{3}H-melatonin were determined using a Packard Minaxi Tri-Carb Series 4000 β–counter. Protein determinations were measured according to the method of Bradford (24).

RT-PCR of sheep tissues for melatonin-related receptor

Sheep tissues were collected from sheep of mixed sex and breed at a local abattoir or from the flock at the Rowett Research Institute (Aberdeen, UK) and frozen on dry ice as quickly as possible. The retina was detached from the RPE by peeling it away from the pigmented RPE after a period 10–15 min of cooling in ice cold ethanol. Messenger RNA was isolated from the sheep tissues and 1 μg used for first strand cDNA synthesis at 42 °C using Superscript reverse transcriptase (Gibco BRL, Paisley, UK) according to the manufacturer's instructions. Control reactions were performed with 1 μg of mRNA as above after RNase or DNase treatment. To ensure that solutions used for reverse transcription were free of contaminating sequences control reactions were processed without the addition of mRNA. Controls consisting of the PCR reaction mixture only were also included for each PCR reaction performed to check that the PCR reaction components and primers did not contain contaminating sequences. PCR was performed on 2 μl aliquots of the 20 μl first strand cDNA reaction using sheep melatonin-related receptor specific oligo primers. The primer pairs used were designed to give amplification between exon 1 and exon 2 of the melatonin-related receptor gene thus eliminating PCR of genomic DNA and consisted of P1MRR 5′-GGTCATTTTGGCTGTGTCGAAGAAC-3′, position 144–168 and P2MRR 5′AAACATGGTTAGAAAATTTCGAA-3′, position 699–721. Hot start PCR was performed using 100 pmol of each primer, 2.5 U of AmpliTaq in the presence of 200 μM dNTPs and 1.5 mM $MgCl_2$ $6H_2O$. The PCR reaction cycles consisted of 94 °C for 1 min, 50 °C for 1 min 30 s and 72 °C for 1 min 30 s for 35 cycles.

The PCR products were electrophoresed through 1% agarose gels and Southern blotted onto GeneScreen nylon filters (NEN Dupont Ltd, Stevenage, Herts, UK) using a pressure blotter (Stratagene). Hybridization was performed at 55 °C in QuikHyb hybridization solution (Stratagene) according to the manufacturer's instructions using an ovine melatonin-related receptor specific oligo probe (5′-ATGGTGGGGTTCATCACAGGCC-3′, position 308–329) internal to the primers used for PCR. The filters were washed to a final stringency of $1 \times$ SSC at 55 °C. PCR products giving a positive hybridization signal were subcloned using the pGEM-T vector system (Promega) and DNA was prepared for sequencing to confirm the identity of the PCR products.

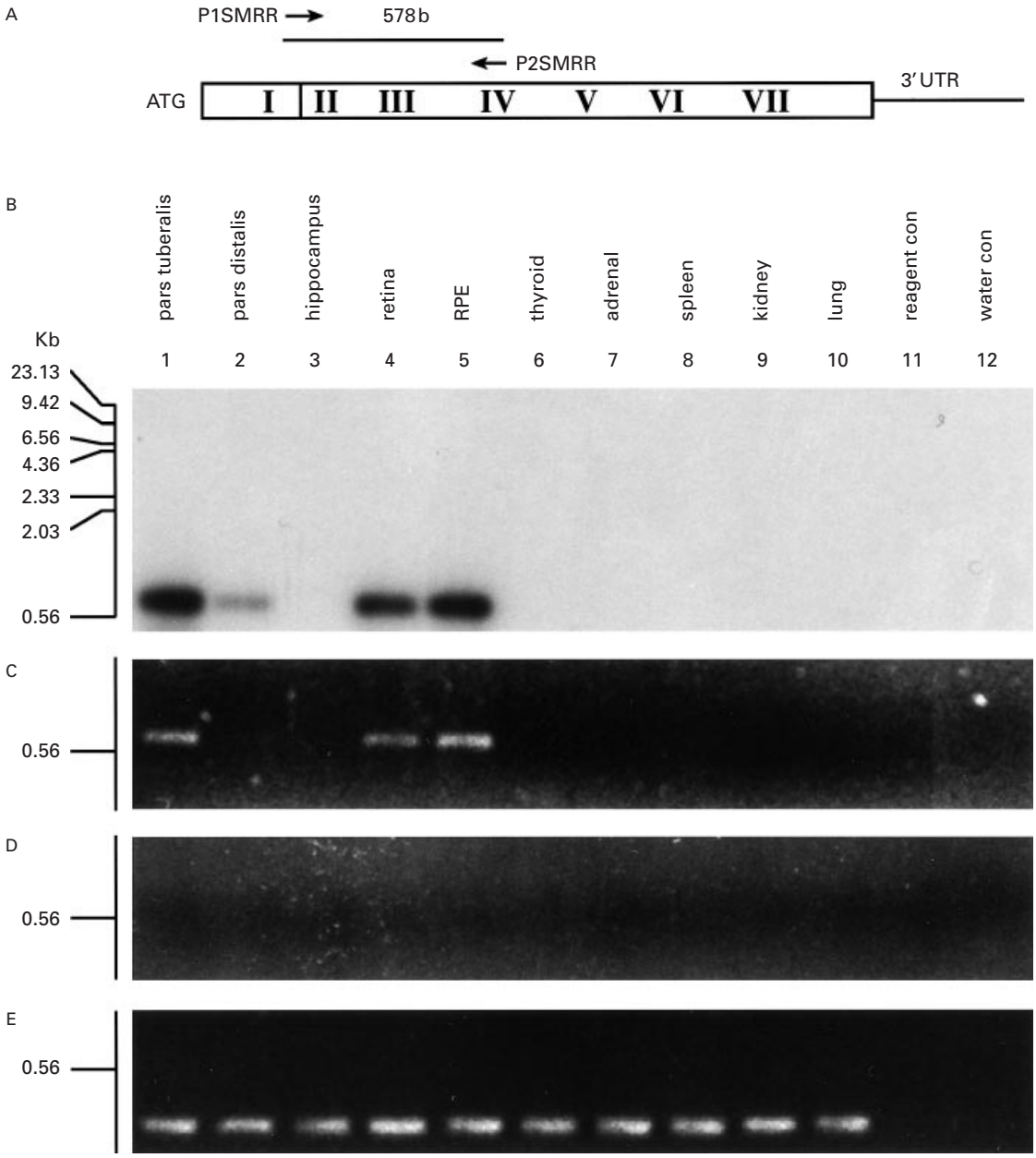

FIG. 6. RT-PCR for melatonin-related receptor gene expression in ovine tissues. (A) Schematic diagram of melatonin-related receptor cDNA showing location of primer sites used for RT-PCR P1SMRR [5′-GGTCATTTTGGCTGTGTCGAAGAAC-3′], position 143–167 and P2SMRR [5′-AAACATGGTTAGAAAATTTCGAA-3′], position 698–720. The size of the PCR product obtained is indicated. The splice site between exon 1 and exon 2 of melatonin-related receptor genes is indicated by the vertical line between transmembrane domains I and II in the diagram. (B) Southern blot of the RT-PCR products hybridized with a specific ovine melatonin-related receptor radiolabeled oligo probe (5′-ATGGTGGGGTTCATCACAGGCC-3′, position 308–329) showed hybridization to products of the appropriate size. (C) RT-PCR products were electrophoresed on an ethidium bromide stained agarose gel for Southern blotting. (D) RNase treated control RT-PCR reactions electrophoresed on ethidium bromide stained agarose gel. (E) Control reactions were performed on the above cDNA samples using primers for the G_s alpha subunit exon 8 sense primer [5′-CGTGTCCTGACTTCTGGAATCTTTGAGACC-3′] and a G_s alpha subunit exon 10 antisense primer [5′CCATCTGTTGTTCCAGATGCTCTT-3′]. A single product of the appropriate size (0.241 kb) was obtained from each tissue cDNA sample.

In situ *hybridization*

Tissue was collected as above and frozen in isopentane chilled over dry ice for *in situ* hybridization. Cryostat sections (10–20 μm thick) of sheep brain, pituitary and retina/RPE were thaw-mounted onto poly D-lysine coated slides and stored at −70 °C until use. The retina and RPE were frozen together and cut in small pieces of around 5 mm for preparation of cryostat sections. The two tissue layers detach and separate on mounting onto the slides and the RPE is clearly visible as a pigmented layer on the slide with an adjacent layer of retinal tissue visible when viewed with a microscope. Sections were then fixed in 4% paraformaldehyde (pH 7.4) in 0.1 M PBS treated with 0.1% diethylpyrocarbonate (PBS-DEPC) for 20 min then rinsed twice with PBS-DEPC followed by acetylation with 0.1 M triethanolamine and 0.25% acetic anhydride for 10 min. After washing twice in PBS-DEPC, sections were dehydrated in a graded series of ethanol-DEPC water solutions before being dried under vacuum for 60 min prior to hybridization with ^{35}S-labelled

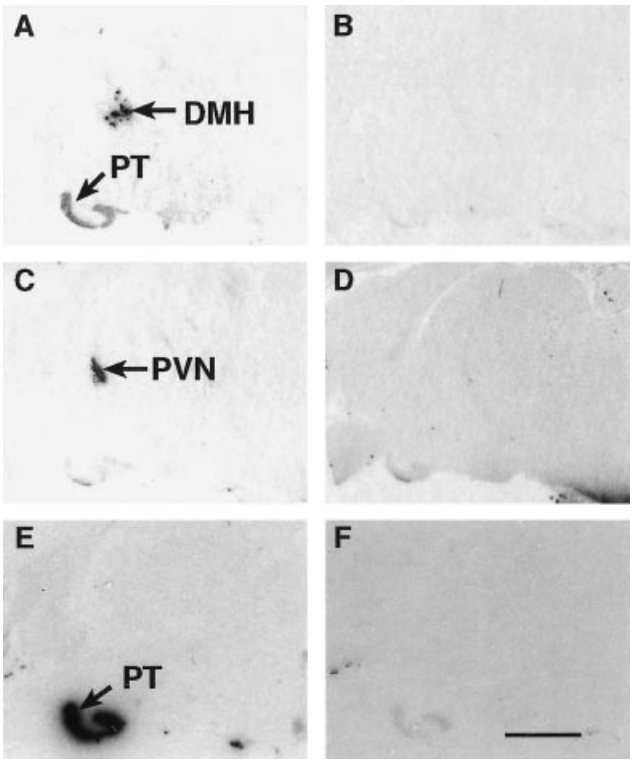

FIG. 7. Localization of expression of melatonin-related receptor mRNA by *in situ* hybridization in sagittal sections of the sheep brain and pituitary. (A) [^{35}S]antisense melatonin-related receptor riboprobe hybridized to the dorsal medial hypothalamus (DMH) and the pars tuberalis (PT). (B) Non-specific hybridization of the melatonin-related receptor sense probe. (C) [^{35}S]antisense corticotrophin releasing factor (CRF) oligo probe hybridized to the paraventricular nucleus (PVN). (D) Non-specific hybridization of the CRF sense oligo probe. (E) Total 2-[^{125}I]iodomelatonin binding to the pars tuberalis and (F) in the presence of 10^{-7} M melatonin. Tissue sections hybridized with [^{35}S]melatonin-related receptor or the CRF oligo were exposed to Hyperfilm β–Max for 7–21 days and tissue sections showing 2-[^{125}I]iodomelatonin binding were exposed to film for 1–3 days. Bar = 0.7 cm.

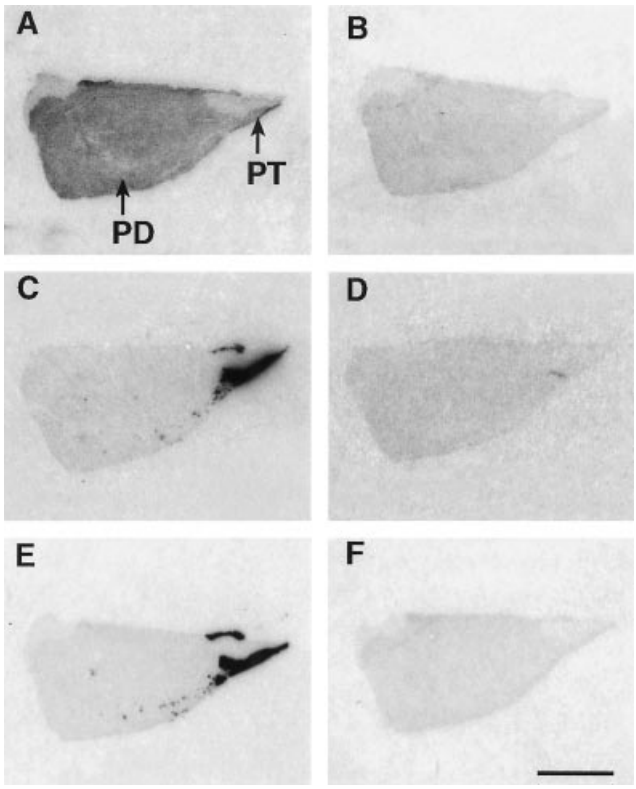

FIG. 8. Expression of melatonin-related receptor mRNA in ovine pituitary. (A) [^{35}S]melatonin-related receptor antisense and (B) [^{35}S]melatonin-related receptor sense riboprobes hybridized to sagittal sections of the pituitary gland. Specific hybridization is present over the pars tuberalis (PT) and pars distalis (PD). (C) Total 2-[^{125}I]iodomelatonin binding over the pars tuberalis of the pituitary gland and (D) in the presence of 10^{-7} M melatonin. (E) [^{35}S]Mel$_{1a\beta}$ specific antisense and (F) Mel$_{1\beta}$ sense riboprobes hybridized to sagittal sections of the pituitary gland. Tissue sections hybridized with [^{35}S]melatonin-related receptor and [^{35}S]Mel$_{1a\beta}$ receptor were exposed to Hyperfilm β–Max for 21 and 7 days, respectively. Tissue sections showing 2-[^{125}I]iodomelatonin binding were exposed to film for 1 day. Bar = 0.5 cm.

riboprobes. ^{35}S-labelled antisense and sense riboprobes were synthesized by *in vitro* transcription using the appropriate RNA polymerase in the presence of ^{35}S-alpha-thio-UTP (NEN; 1000 Ci/mmol). The melatonin-related receptor template consisted of a fragment generated by RT-PCR of sheep genomic DNA using the following primers: 5′-TGGCCATCGGCGGCTGGGATCTCAG-3′, position 267–291 and 5′-AAACATGGTTAGAAAATTTCGAA-3′, position 698–720. The PCR products were subcloned into pGEM-T and the sequence of the riboprobe template verified by DNA sequencing. The AANAT template was kindly supplied by Dr D. C. Klein (National Institutes of Health, Bethesda, MD, USA) and consisted of a 960 bp sheep cDNA fragment short of sequence encoding the *C*-terminal 30 amino acids. A full length Mel$_{1a\beta}$ (13) template was used. Antisense and sense oligo probes against corticotrophin releasing factor (CRF) were designed as reported by Broad *et al.* (14). Alternate pairs of adjacent tissue sections were overlaid with 70 µl of hybridization buffer containing 7×10^3 cpm/µl melatonin-related receptor, AANAT or Mel$_{1a\beta}$ ^{35}S-labelled antisense riboprobe or 5×10^3 cpm/µl CRF ^{35}S-labelled antisense oligo probe. The corresponding adjacent sections were hybridized with the appropriate sense specific riboprobe (7×10^3 cpm/µl) or oligo probe (5×10^3 cpm/µl) to determine levels of background hybridization of ^{35}S-labelled probe to the sheep tissues. The hybridization buffer consisted of 50% formamide, 300 mM NaCl, 1 × Denhardts solution, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% dextran sulphate, 10 mM DTT and 0.5 mg/ml tRNA. Sections were incubated with melatonin-related receptor, AANAT or Mel$_{1a\beta}$ riboprobes at 58 °C and with CRF oligo probes at 45 °C for 16–18 h. Riboprobe hybridized sections were washed four times in 4 × SSC then treated with an RNase A solution consisting of 20 µg/ml RNase A, 500 mM sodium chloride, 10 mM Tris (pH 8.0) and 1 mM EDTA for 30 min at 37 °C. Sections were then washed to a final stringency of 0.1 × SSC at 60 °C. CRF oligo probes were washed twice in 4 × SSC, once in 1 × SSC and a final stringency wash in 1 × SSC at 55 °C. All tissue sections were dehydrated in a graded series of ethanol, air dried and apposed to Hyperfilm-βMax film (Amersham International, UK).

2-[^{125}I]Iodomelatonin in vitro *autoradiography*

Localization of specific 2-[^{125}I]iodomelatonin binding over sheep brain and pituitary glands was determined using 80 pM 2-[^{125}I]iodomelatonin in the presence or absence of 10^{-7} M melatonin as described previously (25).

DNA sequencing

DNA was prepared for sequencing using a Wizard 373A DNA purification kit (Promega) according to the manufacturers instructions. DNA sequencing was performed by the dideoxy-sequencing method of Sanger *et al.* (26) using universal M13 primers and synthetic oligo primers and an Applied Biosystems model 373 DNA sequencer (Rowett Research Institute, Aberdeen, UK) and an Applied Biosystems model 377 DNA sequencer (University of Durham, Durham, UK).

Chemicals and enzymes

All chemical reagents were obtained from Sigma International, UK unless otherwise stated. Restriction and DNA modifying enzymes and enzyme buffers were obtained from Promega, Southampton, UK or Gibco BRL Life

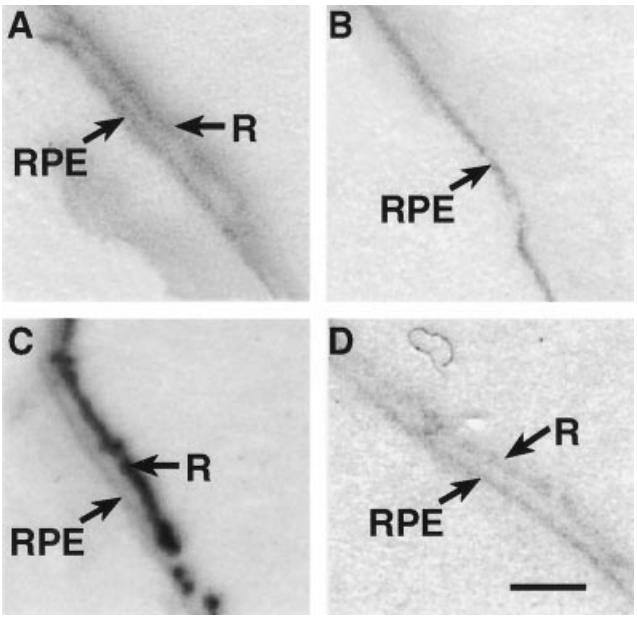

FIG. 9. Expression of melatonin-related receptor mRNA in ovine retina and retinal pigment epithelium. (A) [^{35}S]melatonin-related receptor antisense and (B) melatonin-related receptor sense riboprobes hybridized to sagittal sections of the retina (R) and retinal pigment epithelium (RPE). (C) [^{35}S]AANAT antisense and (D) AANAT sense riboprobes hybridized to sagittal sections of the retina and RPE. Tissue sections hybridized with [^{35}S]melatonin-related receptor and [^{35}S]AANAT were exposed to Hyperfilm β–Max for 21 and 14 days, respectively. Bar = 0.25 cm.

Technologies Ltd, Paisley, UK. Random and synthetic oligo primers, cell culture media and reagents were purchased from GibcoBRL Life Technologies Ltd, Paisley, UK.

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