Partial cloning and polymorphism of the melatonin_{1a} (Mel_{1a}) receptor gene in two breeds of goat with different reproductive seasonality

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The melatonin_{1a} (Mel_{1a}) receptor gene was cloned in two breeds of goat, one with marked seasonal ovarian activity (Alpine breed) and the other with low seasonal variations in ovulatory activity (Creole breed), to determine whether reproductive seasonality is related to the structure of the Mel_{1a} gene. The main part of exon II was amplified by PCR using sheep sense and antisense primers in 17 Alpine and 13 Creole goats, and cDNAs were subcloned and sequenced in both directions. The results indicate the presence of an identical sequence in 12 of the 30 animals, that is, six Alpine and six Creole goats. The greatest similarity in the 784 nucleotides of exon II (primers

excluded) that was obtained was found with ovine Mel_{1a} receptor sequence (98.4%) and the differences consisted of 12 nucleotide and four amino acid changes. The presence of seven mutations compared with the previous reference sequence was observed and their combinations indicated the presence of at least five other alleles; one mutation resulted in a change in one amino acid in three Alpine goats. No difference in allelic distribution was observed between the two breeds. The results indicate that no relationship could be established between the Mel_{1a} receptor gene structure and the expression of seasonality of reproduction in goats.

Introduction

Seasonal reproductive activity for species living in temperate latitudes is characterized by alternating ovulatory and anovulatory periods triggered by annual variations in daylength. Photoperiodic cues are transduced into neuroendocrine signals by melatonin. In sheep, this hormone is secreted during the night by the pineal gland and acts in the premammillary hypothalamus to control the activity of the GnRH (Malpoux *et al.*, 1998). Three specific melatonin receptor subtypes have been cloned and characterized in mammals: Mel_{1a}, Mel_{1b} and MT3 (Reppert *et al.*, 1996; Nosjean *et al.*, 2000). However, in ovine species, only melatonin_{1a} (Mel_{1a}) receptors have been identified. Some polymorphism has been shown in the gene encoding Mel_{1a} receptor in sheep (Barrett *et al.*, 1997; Messer *et al.*, 1997) and in other species including pigs, cattle (Messer *et al.*, 1997) and humans, in which the prevalence of allelic variants for the Mel_{1a} receptor was observed in subjects with sleep disorders (Ebisawa *et al.*, 1999).

Pelletier *et al.* (2000) found new alleles for the ovine Mel_{1a} receptor. A particular allelic form of the Mel_{1a} receptor gene is characterized by the absence of an *MnII* restriction site at position 605 of the coding sequence, leading to '–/–' homozygous genotypes when the corresponding mutation occurs on both chromosomes. When two groups of ewes chosen for their extreme out of season ovulatory capacity were used, an

association between the genotype '–/–' and the occurrence of a seasonal anovulatory period was revealed in a Mediterranean breed, the Merinos d'Arles (Pelletier *et al.*, 2000).

The aim of the present study was to address the question of whether an association such as the one described in sheep could be observed between a specific allelic form of the Mel_{1a} receptor and the expression of seasonality in a different but related species, the goat. For the purpose of this study, two different breeds of goat, Alpine and Creole, which had different patterns of reproductive seasonality under their natural conditions, were used. In temperate latitudes, Alpine goats have a long anovulatory period during the spring and summer (Chemineau *et al.*, 1992), whereas the Creole breed reproduces throughout the year and has continuous spontaneous ovulatory activity in tropical latitudes (Chemineau, 1986). This contrasting reproductive activity of the two breeds provides an opportunity for examining the putative association of differences in the structure of the Mel_{1a} receptor gene on seasonality.

The objectives of the present study were firstly to obtain the sequence of the gene encoding the goat Mel_{1a} receptor, secondly, to analyse the polymorphism of this gene, and thirdly, to examine the putative differences in the nucleotide sequences between the breeds.

Materials and Methods

General

Genomic DNA was prepared from blood samples of either Alpine or Creole goats. The exon II of the Mel_{1a}

receptor was amplified by PCR using sheep primers and cDNAs were subcloned. After sequencing, the sequence of exon II was compared among individuals, between breeds and with other known Mel_{1a} receptor sequences in different vertebrates.

Experimental animals and management conditions

Alpine breed. The experimental animals were part of the flock kept in the Research Centre facilities at Nouzilly, France (47°N). The goats were all female and aged between 3.5 and 9.0 years, with a mean adult weight of 59 kg. The animals were imported into the Centre from private flocks and three of the pairs that were studied were half sisters from their fathers, indicative of a low level of inbreeding. The extent of the seasonality of reproduction in this breed at the time of the experiment was verified by assessing ovarian cyclicity by measuring plasma progesterone at weekly intervals (Terqui and Thimonier, 1974) in 25 female goats from April to January. Sixteen of the 25 animals were used for cDNA preparation and sequencing, and three females in which cyclicity was not studied were also used.

Creole breed. Blood samples were collected from animals that were considered to be unrelated, from private flocks reared under extensive conditions on the Saintes Island, West Indies (Guadeloupe 16°N). Blood samples from 14 adult goats (13 females and one male) were imported and processed in the same way as the Alpine samples to obtain genomic DNA. Mean adult weights of animals were 27 and 38 kg for females and males, respectively. The absence of seasonality in this flock (reported by Chemineau, 1986) was not confirmed in the experimental animals.

All experimental procedures were performed according to authorization A37801 granted by the French Ministry of Agriculture and Fisheries.

Genomic DNA and cDNA preparations

The protocol used to prepare genomic DNA has been described in detail by Pelletier *et al.* (2000) and involved a total of 19 Alpine and 14 Creole goats: 75–150 µg genomic DNA was used for PCR using primers designed from the sheep sequence of the Mel_{1a} receptor published by Reppert *et al.* (1994). Sense primer corresponded to positions 285–304 of the sheep sequence (onset of exon II) to which an *Xba*I restriction site was added to facilitate further subcloning. Antisense primer corresponded to the 1108–1089 position to which a *Hind*III restriction site was added. This antisense primer was located 41 bases before the 3'-end and was similar to that used by Messer *et al.* (1997). Amplifications consisted of 33 PCR cycles using 1 unit thermophilic Taq polymerase (Promega, Charbonnières): denaturation at 94°C for 1 min; annealing at 56°C for the first four cycles and then at 64°C for the other 29 cycles for 1 min; and extension at 72°C for 2 min. A final extension step at 72°C for 8 min completed the PCR procedure.

PCR products were separated by electrophoresis on a 1% (w/v) agarose gel in parallel with a 100 base pair (bp) DNA marker. As it was assumed that the Mel_{1a} receptor sequence of goats was similar to that of sheep, the band of the expected size (840 bp) was cut and purified (Qiaquick Gel Extraction kit; Qiagen, Courtaboeuf).

The cDNA was then subcloned into a pBluescript plasmid (Stratagen, La Jolla, CA). The presence of an insert within bacterial colonies was assessed both by: (i) radioactive primer hybridization to bacterial colonies deposited on to paper filter; and (ii) post-cloning PCR using previous sense and antisense primers minus the restriction sites. DNA was sequenced from both directions using an automatic sequencer (Applied Biosystem, Foster City, CA).

After difficulties in obtaining the full sequence, data from three animals were discarded. Complete data and comparisons were obtained for 17 Alpine and 13 Creole goats.

Nucleotide sequences were compared with the sequence found to be identical in the majority of cases ($n = 12$) using a multiple sequence alignment procedure (Dessen *et al.*, 1990). Any identical base change from the reference sequence that was found at least twice at the same position in at least two different individuals was considered a 'mutation'. Nucleotide sequence was numbered from the onset of the sense primer, although the parts of sequence generated by both primers were not used in the sequence comparisons. Data were further analysed by comparing the caprine Mel_{1a} receptor sequences with the Mel_{1a} receptor sequences in other vertebrates. An enzymatic restriction map was established and mutations that did or did not induce an amino acid change were described.

Results

Nucleotide and protein sequences

The sequence of 784 nucleotides of the caprine melatonin receptor, independent of the primers, obtained in 12 of 30 amplified and cloned cDNAs (Fig. 1), was considered a reference sequence corresponding to the most frequent allele (allele a) in this study. Translation to amino acid sequence is also presented.

Comparisons between other caprine nucleotide sequences and the reference sequence indicated the presence of at least seven mutations. Only one of these mutations resulted (at position 233) in an amino acid change: a glycine to an arginine (Table 1) located within the second intracellular domain (Fig. 1).

These mutations were not found simultaneously but were associated into different combinations leading to the definition of five other alleles that had one to six mutations in a given sequence (Table 2).

The study of the enzymatic restriction map of the reference sequence indicated the presence of seven *Mn*II sites but none of these was shown to be polymorphic. Similarly, none of the seven mutations changed the enzymatic restriction map.

Fig. 1. Partial sequence of cDNA encoding the melatonin_{1a} (Mel_{1a}) receptor and deduced amino acid sequence (single code letter) in goats. Arrows indicate sense and antisense primers. The six putative transmembrane domains inferred from the ovine sequence (Reppert *et al.*, 1994) are shaded in grey. The sequence has been deposited in GenBank under accession number AF419334.

Table 1. Position of mutations, and base and amino acid changes in the melatonin_{1a} (Mel_{1a}) receptor compared with the reference sequence in Alpine and Creole goats

Position of mutations	Base change	Amino acid change
190	C → T	None
233	G → C	Gly → Arg
307	G → T	None
421	T → C	None
424	C → T	None
577	C → T	None
589	C → A	None

Difference in nucleotide sequences according to breed

The most prominent finding of the contribution of each breed for each allele was that the reference sequence was found in both breeds ($n = 6$ per breed; Table 2). The seven mutations were found within the Alpine breed, whereas five of them were present in alleles of the Creole breed. The two other mutations, at positions 233 and 307, were present in three and four Alpine goats, respectively. As a result, the protein sequence was modified in three animals (mutation at position 233) only in the Alpine breed.

The progesterone assays indicated that all 25 Alpine goats that were examined showed a seasonal period of anovulation that varied from 22 to 31 weeks. No relationship was found between the duration of the anovulatory period and the different alleles of the Mel_{1a} receptor (data not shown).

Nucleotide and protein homologies of the caprine Mel_{1a} receptor sequence with the Mel_{1a} receptor sequence in different vertebrates

The highest similarity between the Mel_{1a} receptor sequence was with the ovine sequences (98.4% for both the nucleotidic and proteic sequences) corresponding to 12 and four changes in nucleotides and amino acids, respectively, for the part of exon II that was considered (Table 3).

The similarity of caprine and bovine nucleotide sequences was also high (90%) but was <85% for nucleotide sequence similarity with other studied vertebrate nucleotide sequences. Nucleotide similarity fell by a further 10% when the Mel_{1b} sequences of humans, mice and Siberian hamsters were compared with the present caprine sequence.

Discussion

The results of the present study provide for the first time: (i) a partial nucleotide sequence encoding the Mel_{1a} receptor in the two breeds of goat: this sequence encompasses about 70% of the presumed total encoding sequence; and (ii) a series of six allelic forms resulting from the combination of seven mutations from which one sequence was found to be identical in 12 of 30 animals and was therefore referred to

as the reference sequence. No difference in the distribution of the various allelic forms was found between the two breeds.

The multiple alignment analysis indicated that the nucleotide sequence found to be identical in six Alpine and six Creole goats, considered the reference sequence, is more closely related to the Mel_{1a} receptor subtype of other vertebrates than to the Mel_{1b} subtype. In addition, the caprine sequence is closer to ruminant sequences than to any other vertebrate sequence considered. As expected, the highest similarity was found with the ovine sequence (Reppert *et al.*, 1994), as only 12 nucleotide differences, of the 784 nucleotides obtained, were registered between the two species, corresponding to over 98% similarity.

The sequence analysis of the 30 individual cloned cDNAs made it possible to observe seven point mutations compared with the reference sequence within the limits of the gene studied here. The presence of other mutations in parts of the Mel_{1a} sequence not considered in the present study is also plausible. The seven mutations are associated to form combinations that vary from one to six, demonstrating the presence of at least five other alleles. The mutation at position 233 resulted in a change in one amino acid residue in three of the 30 animals studied. Overall, the identical reference sequence obtained in the two breeds, as well as the small number of mutations in the sequence, indicate that the structure of the Mel_{1a} receptor gene is well conserved within the caprine breeds.

Two sites of interest were conserved in goats. The first site is the *Mnl* site at position 328–331. Considering the phylogenetic proximity between the two species and the physiological similarities in their reproduction activity, the main unexpected finding of the present study was the absence of polymorphism at the *Mnl* site at position 328–331 of the caprine sequence. This site, corresponding to a polymorphic *Mnl* restriction site at position 612–615 in the ovine sequence, is the marker of the specific allele linked to reproductive seasonality in ewes (Pelletier *et al.*, 2000). This finding is supported by the relationship observed between the frequency of this allelic form for the Mel_{1a} receptor and latitudes. This allele is more frequent in breeds from higher latitudes where pronounced differences in daylength according to the season induce a marked anovulatory period (J. Pelletier and M. Migaud, unpublished). Extensive tests are currently in progress in our laboratory to test this hypothesis on other breeds of sheep.

The results from the present study indicate that the same restriction polymorphism site could not be considered a marker for the expression of seasonality in goats.

The second site of interest is the glycine residue at position 195 of the caprine sequence, which is highly conserved in higher vertebrates. This glycine residue was demonstrated to be necessary for melatonin binding on the human Mel_{1a} receptor (Conway *et al.*, 2000; Gubitza and Reppert, 2000).

Sequence analysis has shown some degree of polymorphism in the caprine sequence; however, no relationship

Table 2. Definition of the different alleles in relation to the number and positions of nucleotide mutations compared with the reference sequence (allele a) in Alpine and Creole goats

Allele	Number of mutations	Breed (n)			Position of mutations						
		Alpine	Creole	Total							
a	0	6	6	12	–	–	–	–	–	–	–
b	1	0	1	1	–	–	–	–	–	577	–
c	4	3	0	3	190	–	–	421	424	577	–
d	4	4	6	10	190	–	–	421	424	–	589
e	5	1	0	1	190	–	307	421	424	–	589
f	6	3	0	3	190	233	307	421	424	–	589

Table 3. Nucleotide and protein similarities of the caprine melatonin_{1a} (Mel_{1a}) receptor (reference sequence) with the Mel_{1a} receptor in different vertebrates

Species	Percentage of nucleotide similarities	Percentage of amino acid similarities
Caprine	100.0	100.0
Ovine ^a	98.4	98.4
Bovine ^b	90.0	90.0
Human ^c	84.4	84.7
Murine ^d	79.5	84.2
Siberian hamster ^e	79.2	83.0
Chicken ^f	72.1	78.1

According to GenBank data: ^aU14109; ^bU73327; ^cU14108; ^dU52222; ^eU14110; ^fU31820.

between alleles and the two breeds was observed. This finding indicates that the distinct expression of seasonality in the two breeds under their normal climatic conditions is not related to a major difference in the structure of the Mel_{1a} receptor gene. However, more animals should be studied and the polymorphism of their Mel_{1a} receptor characterized to obtain a representative population for both breeds to verify this conclusion.

Under the environmental conditions in Guadeloupe, Creole goats are normally continuously cyclic; however, they show a sustained anovulatory period when subjected to the photoperiodic regimen of France (Chemineau, 1994), even though their anovulatory period is shorter than that of the Alpine breed or not present in all animals each year. In addition, Alpine female goats undergoing a marked anovulatory period each year in temperate latitudes (Chemineau *et al.*, 1992) also show reproductive seasonality when reared in the tropical latitudes of Guadeloupe (Y. Cognié, personal communication) as well as under the tropical photoperiod conditions of Guadeloupe within a light-proof building (Chemineau *et al.*, 1992). Taken together, these data indicate that the model used in this study might not have been the most suitable to examine the relationship between the structure of the Mel_{1a} gene and the expression of seasonality in goats. An alternative possibility would be to use progesterone assays to select the Creole goats that are never cyclic under temperate photoperiodic environments and those that have a sustained anovulatory period under the same conditions, thereby reproducing the experimental procedure based on two extreme populations used for the

ovine Merinos d'Arles flock. In addition, the characterization of Mel_{1a} receptor in goats was considered, as an association was found between an allele and the expression of the seasonality of reproduction in sheep (Pelletier *et al.*, 2000). However, it is not possible to rule out the possibility that the gene structure of other melatonin receptor subtypes expressed in goats, such as the Mel_{1b} receptor, could be related to the expression of seasonality of reproduction.

In conclusion, the results of the present study provide for the first time a partial sequence as well as some allelic forms of the Mel_{1a} receptor gene from Alpine and Creole goats. In several ovine breeds, a specific allele was found to be associated with the presence of an annual anovulatory period (Pelletier *et al.*, 2000). This relationship could not be observed in the model using two different caprine breeds, indicating a difference between the two species.

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