

# Comparative Analysis of the Prion Protein Open Reading Frame Nucleotide Sequences of Two Wild Ruminants, the Moufflon and Golden Takin

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## Key Words

Species barrier · Wild ruminants · Open reading frame · Prion protein · Scrapie · Transmissible spongiform encephalopathy

## Abstract

The prion protein (PrP) nucleotide sequences of two ruminants were determined in order to elucidate the differences in susceptibility to spongiform encephalopathy agents in each species. The nucleotide sequences of PrP coding regions of the moufflon and the golden takin encompassed 771 bp in length. The PrP gene sequences of the golden takin were closely related to those of sheep with one amino acid difference. The PrP gene sequence of the moufflon was identical to that of sheep. The similarities between the PrP genes of these two animals and sheep imply that the species barriers between these animals are small or non-existent. These PrP genes could be used to establish transgenic mice with higher susceptibility to prion-related diseases.

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## Introduction

The transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative disorders which occur in many mammalian species [1–5]. These are prion diseases caused by the transformation of prion protein (PrP) [6, 7]. TSEs are reported as kuru, Creutzfeldt-Jacob disease and Gerstmann-Straussler-Scheinker syndrome in man [8–10] and as scrapie and bovine spongiform encephalopathy in domestic ruminants [6, 11, 12]. They have caused serious problems in the food industry and in public health. The kudu (*Tragelaphus strepsiceros*), the scimitar horned oryx (*Oryx dammah*) and the moufflon (*Ovis musimon*) are artiodactyl animals, like sheep (*Ovis aries*) and cattle. These wild ruminants were maintained in zoos before they died of TSE [13–15].

The aetiological agents of these ruminant prion diseases are introduced into animal populations via food-stuffs containing processed offal derived from sheep with scrapie [16, 17]. The moufflon is one of four types of primitive sheep, which are of the same genus as domestic sheep. In this study, the PrP nucleotide sequences of two wild ruminants (the moufflon and golden takin), which have not been analyzed so far, were determined. The nucleotide sequences and the deduced amino acid se-

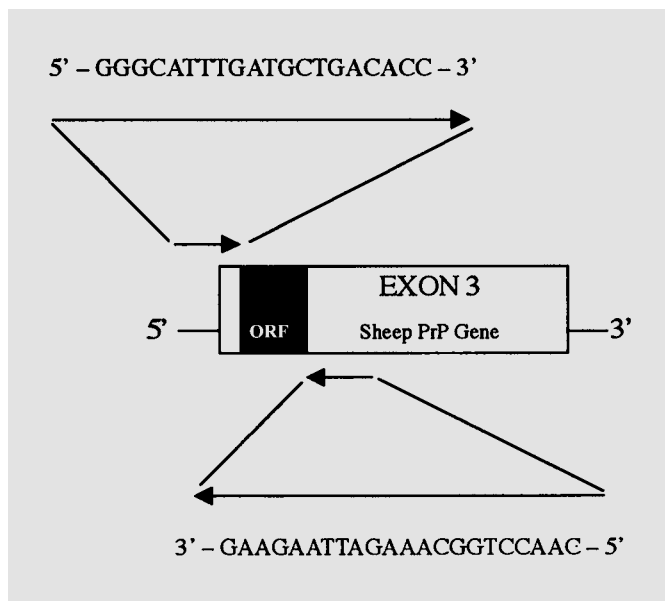
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**Fig. 1.** Construction of a primer pair for DNA-PCR. The primers were designed to amplify a full length of PrP ORF. The closed box indicates sheep PrP coding ORF.

quences were compared with the previously published sheep PrP sequence [18]. The data obtained in this study facilitate the elucidation of the differences in susceptibility to TSE agents in each species.

## Materials and Methods

### Animals

Tissue samples of a moufflon and a golden takin (*Budorcas taxicolor*), none of which had a history of TSE, were obtained from the National Science Museum of Japan.

### DNA Preparation

The shoulder muscles of each animal were homogenized in 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.005 M EDTA. Genomic DNAs, which were isolated from each tissue sample by proteinase K/SDS treatment (0.03% (w/v) in both cases) at 60°, were extracted twice with phenol and subsequently precipitated with ethanol. The concentration of each DNA was estimated by electrophotometric analysis at 260 nm. The resultant genomic DNAs were used as a polymerase chain reaction (PCR) template.

### Preparation of Oligonucleotide Primers for PCR and Sequencing

For PCR amplification and nucleotide sequencing of the PrP genes, 4 oligonucleotide primers were synthesized and used [18] (table 1). The first 2 of these (p294, p295), a primer pair, were designed to amplify a full length of PrP open reading frame (ORF) based on the sequences complementary to the DNA sequences of a sheep PrP genomic clone [18]. Sequences of primers were located approximate-

**Table 1.** Oligonucleotide primers for sequencing

Name	Oligonucleotide primers
p294	5'-GGGCATTGATGCTGACACC3'
p295	5'-CAACCTGGCAAAGATTAAGAAG-3'
p134	5'-CAAGCAGCACACAGTGACCAC-3'
p131	5'-CTGCCACATGCTTCATGTTGG-3'

ly 30 bases upstream and downstream of the PrP gene (fig. 1). These primers were also used for nucleotide sequencing of the PrP gene. The other two primers (p134, p131) were designed to be homologous to an internal region of the PrP ORF of sheep and were used for nucleotide sequencing of the PrP gene.

### DNA-PCR

One microliter (400 g/l) of each of the DNA samples was taken for PCR in 50 µl using a reaction cocktail that encompassed 50 pmol of each of the primers as described above, 0.5 units of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn., USA), 25 mM MgCl<sub>2</sub>, 10 × reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl) and 10 mM dNTPs. The DNAs were amplified for 35 cycles using the GeneAmp PCR System Model 2400 (Perkin Elmer, Chiba, Japan). Cycling conditions were designated at 95° for 1.5 min (denaturation), at 58° for 1.5 min (annealing) and at 72° for 1 min (extension). The PCR products were electrophoresed on a 1.5% agarose gel and the amplified DNA band of approximately 860 bp corresponding to the predicted amplified PrP ORF fragments was excised under UV light and isolated using a Prep-A-Gene DNA purification matrix (Bio-Rad, Richmond, Calif., USA) for cloning.

### Sequencing of PCR Fragments

Each purified DNA fragment was inserted into a pT7 Blue E-vector (Novagen) using a DNA ligation kit (Takara, Shiga, Japan). Plasmid DNA samples were prepared with a Qiagen plasmid mini-kit (Funakoshi, Tokyo, Japan). The nucleotide sequences were determined by the dideoxy chain termination method using a Rhodamine Terminator Cycle sequencing kit (Perkin Elmer, Chiba, Japan) with primers (table 1) in an ABI Prism 310 Genetic Analyzer (Perkin Elmer, Chiba, Japan). The sequence data were processed by means of DNASIS-MAC (Hitachi, Kanagawa, Japan) on a Macintosh personal computer. To avoid potential artifacts induced by PCR [19, 20] and to verify the results, sequencing was performed using three or four independent clones.

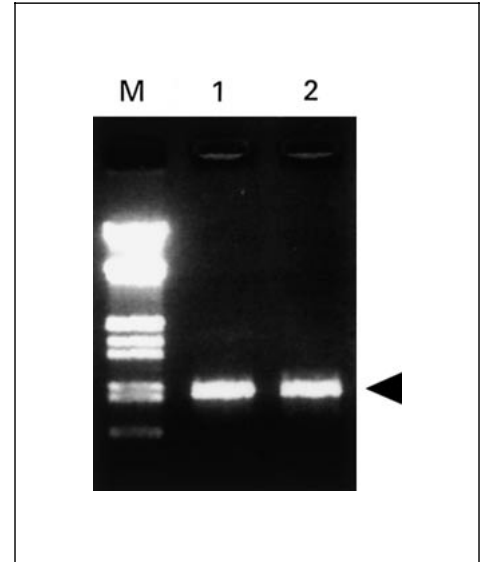
### Comparative Analysis of the Nucleotide and Deduced Amino Acid Sequences

The PrP sequences of moufflon, golden takin, sheep [18], cattle [21] and mice [22] were used for comparative analysis of each of the nucleotide and amino acid sequences.

## Results

### *Amplification of the PrP Genes, and Comparative Analysis of the Nucleotide and Deduced Amino Acid Sequences of the PrP ORF*

Electrophoresis of the PCR products revealed a single PCR product approximately 860 bp in length (fig. 2). The PrP sequences, 771 bp in length, were determined for the moufflon and golden takin (fig. 3). In addition, it was confirmed that they contained the full length of PrP ORF



**Fig. 2.** Amplification of each PrP genomic DNA fragment. Lane 1, the amplified DNA fragment of moufflon PrP ORF. Lane 2, the amplified DNA fragment of golden takin PrP. The arrow indicates the amplified PrP DNA bands (860 bp). M, the molecular weight marker, is a  $\lambda$ HindIII·EcoRI double digest.

sheep	10	20	30	40	50	60	70	80	90	100
moufflon A	ATGGTGAAAA	GCCACATAGG	CAGTTGGATC	CTGGTTCTCT	TTGTGGCCAT	GTGGAGTGAC	GTGGGCCTCT	GCAAGAAGCG	ACCAAAACCT	GGCGGAGGAT
moufflon B	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
golden takin	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
sheep	110	120	130	140	150	160	170	180	190	200
moufflon A	GGAACACTGG	GGGGAGCCGA	TACCCGGGAC	AGGGCAGTCC	TGGAGGCCAAC	CGCTATCCAC	CTCAGGGAGG	GGGTGGCTGG	GGTCAGCCCC	ATGGAGGTGG
moufflon B	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
golden takin	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
sheep	210	220	230	240	250	260	270	280	290	300
moufflon A	CTGGGGCCAA	CCTCATGGAG	GTGGCTGGGG	TCAGCCCCAT	GGTGGTGCT	GGGGACAGCC	ACATGGTGGT	GGAGGCTGGG	GTCAAGGTGG	TAGCCACAGT
moufflon B	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
golden takin	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
sheep	310	320	330	340	350	360	370	380	390	400
moufflon A	CAGTGGAAAC	AGCCACAGTAA	GCCAAAAACC	AACATGAAGC	ATGTGGCAGG	AGCTGCTGCA	GCTGGAGCAG	TGGTAGGGGG	CCTTGGTGGC	TACATGCTGG
moufflon B	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
golden takin	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
sheep	410	420	430	440	450	460	470	480	490	500
moufflon A	GAAGTGCCAT	GAGCAGGCCT	CTTATACATT	TTGGCAATGA	CTATGAGGAC	CGTTACTATC	GTGAAAACAT	GTACCGTTAC	CCCAACCAAG	TGTACTACAG
moufflon B	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
golden takin	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
sheep	510	520	530	540	550	560	570	580	590	600
moufflon A	ACCAGTGGAT	CAGTATAGTA	ACCAGAACAA	CTTTGTGCAT	GACTGTGTCA	ACATCACAGT	CAAGCAACAC	ACAGTCACCA	CCACCACCAA	GGGGGAGAAC
moufflon B	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
golden takin	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
sheep	610	620	630	640	650	660	670	680	690	700
moufflon A	TTCACCGAAA	CTGACATCAA	GATAATGGAG	CGAGTGGTGG	AGCAAATGTG	CATCACCCAG	TACCAGAGAG	AATCCCAGGC	TTATTACCAA	AGGGGGGCAA
moufflon B	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
golden takin	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
sheep	710	720	730	740	750	760	770			
moufflon A	GTGTGATCCT	CTTTTCTTCC	CCTCCTGTGA	TCCTCCTCAT	CTCTTCTCTC	ATTTTTCTCA	TAGTAGGATA	G		
moufflon B	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
golden takin	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

**Fig. 3.** Alignment of nucleotide sequences of the PrP genes. The nucleotide sequences of the PrP ORF from sheep, moufflon A (AB060288), moufflon B (AB060289) and golden takin (AB060290) were compared with the previously published sheep PrP ORF sequence [18]. Dots indicate the same nucleotides.

sheep	10	20	30	40	50
mouflon	MVKSHIGSWI	LVLVAMNSD	VGLCKRPPK	GGGWNTGGS	YPPQGSPGGN
golden takin	.....	.....	.....	.....	.....
sheep	60	70	80	90	100
mouflon	RYPPQGGGGW	GQPHGGWGQ	PHGGGWQPH	GGGWGQPHG	GGWGQGGSHS
golden takin	.....	.....	.....	.....	.....
sheep	110	120	130	140	150
mouflon	QWNKPSKPKT	NMKHVAGAAA	AGAVVGLGG	YMLGSAMSRP	LIHFGNDYED
golden takin	.....	.....	.....	.....	.....S.....
sheep	160	170	180	190	200
mouflon	RYYRENMYRY	PNQVYYRPVD	QYSNQNNFVH	DCVNITVKQH	TVTTTTKGEN
golden takin	.....	.....	.....	.....	.....
sheep	210	220	230	240	250
mouflon	FTETDIKIME	RVVEQMCITQ	YQRESQAYYQ	RGASVILFSS	PPVILLISFL
golden takin	.....	.....	.....	.....	.....
sheep	256				
mouflon	IFLIVG*				
golden takin	.....				

**Fig. 4.** Alignment of deduced amino acid sequences from the PrP genes. The deduced amino acid sequences from the PrP genes of sheep, moufflon and golden takin were compared with the previously published sheep PrP ORF amino acid sequence [18]. Dots indicate the same amino acid residue.

consisting of 256 amino acids like sheep PrP (fig. 4). There was a 2-base difference in the PrP ORF nucleotide between the golden takin and sheep. As to the moufflon, two types of nucleotide sequences were determined based on heterogenote analysis: one was identical to that of sheep, while the other differed by a 2-base substitution (moufflon A and moufflon B in fig. 3). Several differences encoded amino acid substitution; position 437 was substituted as A–G in the golden takin. The differences seen in moufflon nucleotide sequences resulted in synonymous codons in the amino acid sequences. The deduced amino acid sequences of moufflon PrP were identical to that of sheep. The deduced amino acid sequences of golden takin PrP ORF were closely related to that of sheep with a difference of only one amino acid between the two proteins: Asn–Ser at position 146 (fig. 4). The DNA and protein sequences of the sheep PrP gene shared high homology with those of moufflon A (100% at the nucleotide and 100% at the protein levels), moufflon B (99.7%/100%), and golden takin (99.7%/99%), respectively. In addition, the nucleotide sequence of the bovine PrP cDNA [21] showed approximately 94% identity to those from sheep, moufflon A, moufflon B, and golden takin, respectively. Alignment of bovine PrP and those of sheep, moufflon A, moufflon B, and golden takin revealed 94, 94, 94, 95% amino acid identity, respectively.

## Discussion

The prion hypothesis suggests that the species barrier is due to a different amino acid structure in the protein and the derived prions are solely products of the host genotype [2]. Attempts to modify the species barrier by sensitizing a new host via exposure to the old host before inoculation met with no success. The presence of tissue from a different species might cause immunological effects upon inoculation, though it would be unlikely to cause a large species barrier effect on passage in another species; however, a small effect would be induced upon passage in a subsequent species. This effect might be observed if a host protein formed part of the infectious agent. The more closely related the two species, the smaller the species barrier. The effect would be specific and would only affect the first passage in a different individual of another species [23].

The PrP gene of the golden takin was closely related to that of sheep despite the differences in each genus according to the result of ORF amino acid sequences. The similarities of these species mean that the species barrier between the golden takin and sheep is negligible or non-existent. As for the moufflon, the PrP amino acid sequences were identical to those of sheep, implying that the species barrier between moufflon and sheep was non-existent. This, however, is reasonable because the moufflon is one of four types of primitive sheep, which belong to the same genus as the domestic sheep [24]. The genera close to *O. aries* appear to have PrP genes highly homologous to sheep PrP genes. Although there has not been any TSE report on the golden takin, the disease may occur in this species in the future.

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