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Coordinate regulation of bovine prion protein gene promoter activity by two Sp1 binding site polymorphisms

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ABSTRACT

Relationships between insertion/deletion (Ins/Del) polymorphisms of the bovine prion protein gene (*PRNP*) promoter and bovine spongiform encephalopathy (BSE) susceptibility have been reported. Our previous study has shown that polymorphisms of −6C → T included in the specific protein 1 (Sp1) site in the 5′-flanking region of bovine *PRNP* influence the promoter activity of bovine *PRNP*. The present study shows that 12 and 23 bp Ins/Del polymorphisms in the upstream region and an additional polymorphism (−47C → A) in the Sp1 binding site coordinately affect the promoter activity. Reporter gene assays demonstrated that the bovine *PRNP* promoter containing −47A and 23 bp Del/12 bp Ins or 23 bp Ins/12 bp Ins showed lower promoter activity compared with other haplotypes (23 bp Del/12 bp Ins or 23 bp Ins/12 bp Del with −47C) or the wild-type haplotype (23 bp Del/12 bp Del with −47C). Furthermore, gel shift assays showed that the binding activity of Sp1 to the *PRNP* promoter was influenced by both polymorphisms with corresponding effects on the promoter activity. The coordinate regulation of the bovine *PRNP* promoter suggests the two Sp1 binding site polymorphisms control Sp1 binding to the *PRNP* promoter and its activity.

The level of prion protein (PrP) is known to influence the initiation and progression of prion diseases, a group of fatal transmissible neurodegenerative disorders that include kuru and Creutzfeldt–Jakob's disease in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle [1]. Incubation periods following experimental inoculation with prions are known to be inversely proportional to the PrP expression level in transgenic mice over-expressing syrian hamster, mouse, or human PrP [2,3,5]. These findings are also supported by the fact that PrP-null mice that do not express PrP are completely resistant to infection with prions [3] whereas heterozygous null mice, with 50% of the normal PrP expression level, have a protracted incubation time and a prolonged duration of illness following prion challenge compared to wild-type animals [3].

Therefore, it might be expected that cattle with higher levels of PrP expression, for whatever reason, would have shorter incubation periods following the ingestion of meat or bone meal from scrapie-infected sheep or BSE-infected cattle or environmental exposure to BSE. While other factors may be relevant to the age distribution of these cases, it is possible that they may represent a group with high natural PrP expression levels resulting in unusually short incubation periods. While such factors may reside in other genetic loci or involve various environmental influences on PrP expression, we consider it worthwhile to investigate if these cases have shared polymorphic variations of bovine prion protein gene (*PRNP*) that affect PrP expression. In cattle, none of the known polymorphisms within the bovine *PRNP* coding sequence seems to have an influence on BSE susceptibility. Until now, the incidence of BSE in 32 European Friesian and 3 Japanese Black cattle has been reported in Japan. It was speculated that the promoter region of the *PRNP* gene might influence the expression level of the protein and thus the incubation period of transmissible spongiform encephalopathies (TSEs) [4]. As a major first step towards identifying the mechanisms regulating PrP expression, we have conducted sequence and functional analysis of the 5′-flanking region of this gene.

Materials and methods

DNA samples. Fat tissues of 44 Japanese Black cows (JB1–JB45) were obtained from the Shirakawa Institute of Animal Genetics (Fukushima, Japan). At present, there are no known hereditary connections between JB1–JB45 and the three Japanese Black cows that were infected with BSE in Japan. Genomic DNA was isolated from the fat tissue samples by phenol/chloroform extraction.

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Table 1
Primer pairs used for PCR in this study

Name forward primer	Sequence forward primer	Name reverse primer	Sequence reverse primer	Product size (bp)	Genomic region[a]
BovPRNP−1634F_BglII	AGAGATCTTAAGTGACTGAACTAATTCCA	BovPRNP−1470R_Hind III	TCAAGCTTTAGCAACTACTCCAAAACTTA	165	47796–47960
BovPRNP−463F_HindIII	AGAAGCTTGAGCAGGAACTGAGTAAATGACGG	BovPRNP+400R_MluI	TAACGCGTCTACCGGTGCGATTCGCCCAT	864	48967–49830
BovPRNP+2389F_MluI	ATAATTACGCGTTGAGTAGATTCATTAGTGGTT	BovPRNP+2526R_BglII	TCAGATCTGGTTGAAACTGTTCAGTT	138	5l819–5l956
PRNP49686_F[b]	TTACCCTCCTGGTTAGGAG	PRNP49777_R[b]	CTAGATTCCTACACACCAC	91/103	49686–49777
PRNP47784_F[b]	GTGCCAGCCATGTAAGTG	PRNP47883_R[b]	TGGACAGGCACAATGGG	100/123	47784–47883

[a] Nucleotide position refer to AJ298878.
[b] Primer referenced from Hills et al. [20].

DNA amplification. Three parts of the bovine *PRNP* promoter region were amplified by polymerase chain reaction (PCR) (Table 1). The 864-bp fragment, which included exon 1, upstream of exon 1, and the first part of intron 1, was amplified using primers BovPRNP−463F_HindIII and BovPRNP+400R_MluI. For the 165-bp fragment, which included the upstream and downstream regions of position −1594 indel 23 bp, primer pair BovPRNP−1634F_BglII and BovPRNP−1470R_Hind III was used. A 138-bp fragment, which included the last part of intron 1 and the first part of exon 2, was amplified using primer pair BovPRNP+2389F_MluI and BovPRNP+2526R_BglII. Forward and reverse primer pairs were synthesized from reference nucleotide sequence AJ298878 (GenBank from European Friesian) [6]. PCR was performed in a 50 μl reaction volume containing 200 ng DNA, 2.5 U Taq polymerase (Applied Biosystems, Norwalk, CT, USA), 50 pmol of each primer, 2.5 mM dNTPs (Applied Biosystems), 2 mM $MgCl_2$, and the reaction buffer provided by the manufacturer. The amplification was performed using an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, the indicated annealing temperature for 2 min, and extension at 72 °C for 1 min. A final extension step was included at 72 °C for 10 min. All PCR products were electrophoresed on 1% Sea Kem® GTG® agarose gels (FMC BioProducts, Rockland, ME, USA). For the genotyping of the 12 or 23 bp indel polymorphisms, smaller products within the indicated regions were generated from 44 Japanese Black cattle (Table 1) using primer pairs PRNP49686_F and PRNP49777_R or PRNP47784_F and PRNP47883_R. PCR were performed as described previously by Sander et al.[6].

DNA sequencing and construction of plasmids for promoter analysis. The 864, 165, and 138 bp PCR products were cloned into vector pT7Blue-T (Novagen, Madison, WI, USA) and sequenced on both strands using primers −21M 13 forward and M13 reverse with a ABI Prism® Big Dye terminator cycle-sequencing ready kit (Applied Biosystems). Products of the sequencing reaction were run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Then, the resultant products were subcloned into the luciferase reporter gene pGL3-basic (Promega, Madison, WI, USA).

Cell culture. CKT-1 (bovine fibroblast-like epithelial cell) cells were kindly provided by Professor Motohiro Horiuchi, Hokkaido University, Japan. The cell line was grown in Eagle's minimum essential medium supplemented with 5% fetal calf serum (FCS). Neuroblastoma cells (N2a) were cultured in Eagle's minimum essential medium with nonessential amino acids, sodium pyruvate, and supplemented with 10% fetal calf serum at 37 °C under 5% CO_2 for the luciferase assay.

Transient transfection luciferase assay. For the transient transfection assays, 5×10^4 CKT-1 cells or 6×10^4 N2a cells were seeded 48 h before transfection into 24-well plates. Cells reaching 60–80% confluency were transfected with 540 ng/well for each test construct using vector pRL-SV (60 ng/well). Luciferase activity of the cell lysates prepared at 48 h after transfection was measured as relative light units with the Fluoroscan Ascent FL (Labsystems, Franklin, MA, USA) using the Dual-Luciferase Assay System (Promega). Relative luciferase activities were defined as the ratio of the firefly luciferase/renilla luciferase mean value of each construct related to the pGL3-control Vector (Promega), which contains the SV40 promoter.

Gel shift assay. Nuclear extracts were prepared from N2a using the Nuclear Extraction Kit (CHEMICON International, Inc. USA). DNA fragments were isolated from plasmids DelDel, DelIns, DelIns-Sp1, InsIns, InsIns-Sp1, and InDel, which were used in the luciferase assay. These plasmids were cut with BglII, and purified from a 1.5% Sea Kem® GTG® agarose gel (FMC BioProducts, Rockland, ME, USA) using a GEL EXTRACTION SYSTEMS Kit (Marligen, Bioscience Inc., USA). DNA fragments (50 ng) were incubated with 0.1 μg of nuclear extract for 30 min at room temperature in a 10 μl binding reaction that contained 4 μl 5× binding buffer [250 mM NaCl, 50 mM Tris–HCl, pH 7.5, 2.5 mM EDTA, 2.5 mM dithiothreitol, 5 mM $MgCl_2$, 20% glycerol]. Following incubation, 2 μl of 6× EMSA gel-loading solution (Invitrogen, Molecular Probes, USA) was added and the samples were then electrophoresed at 4 °C on a 4% (*N*,*N*′-methylenebisacrylamide is included at 1/40 the concentration of acrylamide) polyacrylamide, non-denaturing gel in 0.5× Tris–boric acid-electrophoresis (TBE) buffer. Then, the gel was stained by SYBR® Green EMSA staining solution according to the manufacturer's protocol (Invitrogen, Molecular Probes, USA).

Results

Analysis of the 12 and 23 bp Ins/Del polymorphisms of *PRNP* gene in Japanese Black cattle was performed and 44 samples were genotyped (Table 2). Representative electropherograms for the 12 and 23 bp indel polymorphisms are shown in Fig. 1. DNA agarose gel electrophoresis showed that Japanese Black cattle homozygous for the 12-bp insertion or deletion showed one thick band, at 91 or 103 bp, respectively (Fig. 2A). The 12-bp deletion was homozygous in 43.2% of Japanese Black cattle, which was higher than the rate of the homozygous 12-bp insertion (15.9%) (Table 2). Similarly, the 23-bp deletion was homozygous in 47.7% of Jap-

Table 2
Genotype frequency of polymorphisms in the putative promoter and intron 1 of the bovine *PRNP* in Japanese Black cattle

Polymorphism	Total	+/+	+/−	−/−
Promoter (23 bp indel[a])	44	0.091	0.432	0.477
Intron 1 (12 bp indel[b])	44	0.159	0.409	0.432
		C/C	C/A	A/A
Spl binding site (SNP[c])	44	0.989	0.011	0

[a] TCTCAGATGTCCTCCCAACAGCA.
[b] GGGGGCCGCGGC.
[c] Polymorphism at nucleotide 49383; SNP, single nucleotide polymorphism.