Studies on the promoter region of the bovine prion protein gene (*PRNP*)

（ウシプリオン蛋白遺伝子プロモーター領域に関する研究）

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CONTENTS

Pages

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Chapter 1

Novel single nucleotide polymorphisms in SP1-binding site of Bovine *PRNP* promoter in Japanese Black cattle: Impairment of its promoter activity

Chapter 2

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

Chapter 3

Investigation of PrP overexpression to the bovine *PRNP* promoter region

ABBREVIATIONS

ANOVA	analysis of variance
bp	base pair(s)
BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
GPI	glycosyl phosphatidyl inositol
NEA	non-essential amino acids
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBS-T	PBS containing 0.1% Tween-20
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride

PrP	prion protein
PRNP	prion protein gene
PrP^{C}	cellular isoform of prion protein
PrP^{Sc}	scrapie isoform of prion protein
SDS	sodium dodecyl sulphate
SNPs	single nucleotide polymorphisms
Sp1	specificity protein 1
TSE	transmissible spongiform encephalopathy

GENERAL INTRODUCTION

The transmissible spongiform encephalopathies (TSEs) or prion diseases, comprise a group of slow degenerative diseases of the central nervous system (CNS), characterized by gliosis, loss of neurons and the formation of the amyloid plaques (Prusiner, 1987). TSEs, which include Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI) in humans, bovine spongiform encephalopathy in cattle, and scrapie in sheep and goats (Prusiner, 1998), is known to be associated with alterations in the neural membrane protein, prion protein (PrP) (Oesch et al., 1985; Hope et al., 1991).

The agent responsible for transmission of the disease is thought to be a protein called PrP^{Sc} [which stands for the scrapie isoform of the PrP] (Bolton et al. 1982; Prusiner, 1982). PrP^{Sc} is derived through an ill-defined post-translational process involving conformational changes from the normal cellular isoform of the prion protein (PrP^{C}). PrP^{Sc} can be distinguished from PrP^{C} by its high content in beta-sheet structure and its partial resistance to digestion with proteinase K (Oesch et al., 1985; Pan et al., 1993). Tow models have been proposed to explain the conversion of PrP^{C} to PrP^{Sc}. On the one hand, the refolding model postulates that PrP^{C} must be partially unfolded and refolded under the direction of PrP^{Sc} as a template (Prusiner, 1991). On the other hand, the nucleation model implies a partly flexible conformation of PrP^{C} which adapts to the

conformation of a PrP^{Sc} polymer after binding to the latter, with the polymer thus acting like a seed (Caughey et al., 1997). The models of mechanisms by which PrP^{C} is converted into PrP^{Sc} remain unknown but clearly, the infectious character of PrP^{Sc} and its ability to propagate absolutely requires the presence of normal PrP^{C}. This has been evidenced by the fact that mice in which *Prnp* gene has been deleted remain resistant to infectivity and toxicity brought by pathogenic inoculates (Büeler et al.1993, Brandner et al., 1996). Furthermore, elevated *Prnp* gene dosage and expression has been established that it associates with a lethal neuromyopathy (Westaway et al., 1994).

Therefore, the prion gene plays a central role in the TSEs. Certain TSE host genetic factors modulate the susceptibility to prion infection (Scott et al., 1999). This phenomenon was initially discovered in sheep, where several mutations within the coding sequence of the prion protein gene (*PRNP*) are known to lead to increased or decreased scrapie susceptibility (Belt et al., 1995; Bossers et al., 2000; Hunter et al., 1992; Hunter et al., 1994; Laplanche et al., 1993). Similar genetic differences exist in humans, where *PRNP* polymorphisms have controversially (Mitrova et al., 2005) been reported to modify the development of CJD (Owen et al., 1990; Collinge et al., 1991; Palmer et al., 1991; Deslys et al., 1994; Alperovitch et al., 1999). However, the known coding polymorphisms within the bovine *PRNP* gene have little or no effect on BSE susceptibility in cattle (Jürgen et al., 2008). It was speculated that the promoter region

of the gene might influence the expression level of the protein and thus the incubation period of TSEs (Bossers et al., 1996). However, the promoter region still remains unclear.

In this study, normal *PRNP* promoter region polymorphisms in Japanese Black cattle, regulation of promoter activity and relationship between transcription factors were investigated.

Chapter 1

Novel single nucleotide polymorphisms in SP1-binding site of Bovine *PRNP* promoter in Japanese Black cattle: Impairment of its promoter activity

1.1. SUMMARY

Susceptibility to transmissible spongiform encephalopathy and different alleles of the prion protein gene (*PRNP*) of humans and sheep are associated. A tentative association between *PRNP* promoter polymorphisms and bovine spongiform encephalopathy (BSE) susceptibility has been reported in German cattle, whereas none of the known polymorphisms within the bovine *PRNP*-coding sequence affects BSE susceptibility. In the present study, novel single nucleotide polymorphisms (SNPs) located in the 5' flanking region of Bovine *PRNP* affecting its expression were demonstrated in Japanese Black cattle. We sequenced exon1 and the approximately 200-bp 5' flanking region of the *PRNP* translation-initiation site containing the proximal promoter of *PRNP* was harvested. We identified 7 SNPs; -184A>G, -141T>C, -85T>G, -47C>A, -6C>T, +17C>T and +43C>T. Six segregated haplotypes in the population were cloned into luciferase-expressing plasmids, transfected into N2a cells and their reporter activities were measured at 48 h post-transfection. Six haplotypes showed decreased expression level in some haplotypes (including -6C>T in Sp1-binding site ($p<0.05$) or -141T>C ($p<0.01$) at 48 h) compared with the wild-type haplotype. These results advocate that certain polymorphisms such as Sp1-binding site polymorphisms in bovine *PRNP*-promoter region in Japanese Black cattle could influence promoter activity, suggesting that

breeding cattle with such substitutions may be a useful approach in reducing BSE risk.

1.2. INTRODUCTION

Bovine spongiform encephalopathy (BSE) belongs to a category of transmissible spongiform encephalopathies (TSE) characterized by neuronal vacuolation and accumulation of an abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system (CNS) (Prusiner, 1987). The PrP^{Sc}, which is converted from the cellular isoform of prion protein (PrP^{C}), is infectious. It is generally believed that BSE is cause by the ingestion of meat and bone meals from scrapie-infected sheep or BSE-infected cattle (Wilesmith et al., 1988). BSE poses a threat not only to cattle but also to humans, since the new variant CJD (vCJD) in humans is probably the result of an infection with BSE prions, which are contained in meat products from BSE-infected cattle (Bruce et al., 1997; Scott et al., 1999). Consequently, many countries are developing policies aimed at eliminating BSE-affected animals from the food chain (Onodera et al., 2006). One such strategy is the breeding of BSE-resistant cattle lines. Certain TSE host genetic factors modulate the susceptibility to prion infection (Scott et al., 1999). This phenomenon was initially discovered in sheep, where several mutations within the coding sequence of the prion protein gene (*PRNP*) are known to lead to increased or decreased scrapie susceptibility (Belt et al., 1995; Bossers et al., 2000; Hunter et al., 1992; Hunter et al., 1994; Laplanche et al., 1993). Similar genetic differences exist in humans, where *PRNP* polymorphisms have controversially (Mitrova

et al., 2005) been reported to modify the development of CJD (Owen et al., 1990; Collinge et al., 1991; Palmer et al., 1991; Deslys et al., 1994; Alperovitch et al., 1999). Thus, identifying genetic variations correlated with TSE resistance is an important step to eliminate TSE from the food chain.

A few *PRNP* polymorphisms have been characterized in cattle. Bos taurus in GenBank shows five nucleotide differences, including insertion/deletion polymorphisms within the coding sequence of the *PRNP*. The polymorphisms in cattle are not significantly correlated with BSE (Hunter et al., 1994; Neibergs et al., 1994; Hernandez-Sanchez et al., 1994; Nakamitsu et al., 2006). Recently a tentative association between *PRNP*-promoter polymorphisms and BSE susceptibility has been found in German cattle (Sander et al., 2004). Furthermore, polymorphisms found in *PRNP* promoter region affect the promoter activity of Holstein Friesian cattle in Japan (Inoue et al., 1997). Very recently, one paper has reported polymorphisms of *PRNP* in Japanese Black, which accounted for approximately 95 % of cattle strain bred in Japan (Nakamistu et al., 2006). The 5' non-coding region of the bovine *PRNP*, resembling genomic structures of the mouse, rat and sheep *PRNP*, consists of three exons. This 3-exon region actually contradicts the two exons found in the hamster and human *PRNP* (Basler et al., 1986; Puckett et al., 1991; Saeki et al., 1996; Westaway et al., 1994). For the bovine gene, a major promoter activity has been identified within the 5'-flanking

region from -88 and -30, which contains only one putative Sp1 (specificity protein 1)-binding site (Inoue et al., 1997). Sp1 is a sequence-specific transcription factor that binds the GC box and activates a wide range of viral and cellular genes (Suske et al., 1999). Sp1 sites have been shown to be functional in many housekeeping gene promoters, where the transcription factor acts as a strong activator (Anderson et al., 1991). Therefore, the potential Sp1-binding site could be functional in the bovine *PRNP* in collaboration with the transcription factors. Potential Sp1-binding site polymorphisms may induce alterations in the allele-specific expression level and/or expression site (Hills et al., 2001). Although the function of the potential promoter region of the bovine *PRNP* has been documented (Inoue et al., 1997), the influence of Sp1-binding site polymorphisms on activity of the *PRNP* promoter region remains unclear.

In this study, normal *PRNP* promoter region polymorphisms, parts of the bovine *PRNP* promoter region, were initially analyzed in Japanese Black cattle breeds. Variations of the bovine *PRNP*-promoter region in 45 Japanese Black cattle, including three potential Sp1-binding sites and exon 1, were elucidated. Activities of the bovine *PRNP*-promoter region and the relevant promoter region were then analyzed. Certain polymorphisms in the bovine *PRNP*-promoter region of Japanese Black cattle were found, and variation of -6C>T in Sp1-binding sites or -141T>C resulted in an

approximately 20% decrease of luciferase activity compared with variants at other positions or the non-substituted *PRNP*-promoter region.

1.3. MATERIALS AND METHODS

DNA samples

Fat tissues of 45 Japanese Black cows (JB1-JB45) were obtained from Shirakawa Institute of Animal Genetics (Fukushima, Japan). Genomic DNA was isolated from the fat tissue by phenol/chloroform extraction. Briefly, bovine tissue samples were dissected and treated with 50 ml of DNA extraction buffer (150 mM NaCl, 10 mM Tris-HCl, 10 mM EDTA-2Na, 0.1 % SDS) containing proteinase-K (100 μg/ml). Mixtures were incubated at 55°C for 1 h and at 37°C for 16 h before treatment with an equal volume of neutral phenol. The mixture was agitated gently for 20 min before centrifugation at 2,000 g for 10 min at room temperature (RT). Supernatants were further treated with an equal volume of PCI (phenol/chloroform/isoamylalcohol in the vol/vol ratio of 25 : 24 : 1) solution and subjected to gentle mixing for 20 min before centrifugation at 2,000 g for 10 min at RT. The isolated supernatant was gently mixed with two volumes of ethanol, and incubated at -80°C for 10 min before centrifugation (10,000 g for 10min at RT). The supernatant was discarded and the pellet washed with 70% ethanol. Dried DNA pellets were dissolved appropriately with Tris-EDTA (TE) buffer.

DNA amplification

A 546-bp fragment of the bovine *PRNP*-promoter region, which included exon1 and upstream-downstream of exon1, was amplified by PCR (Fig.1). Forward and reverse primer pairs were synthesized from reference nucleotide AJ298878 (Gene Bank) [28]. Two oligonucleotides, BPrP-463F (5'-AGAAGCTTGAGCAGGAACTGAGTAAATGACGG-3') and BPrP+83R (5'–TGGGAGTTTAAAGGACTACGCGGC-3'), were used as primers to amplify the base-pairs -463 to +83 of the gene (Fig. 1) in PCR. The PCR was performed in a 50-μl reaction volume containing 150 ng DNA, 2.5 U Taq polymerase (Applied Biosystems, Norwalk, CT, USA), 50 pmol of each primer, 2.5 mM dNTPs (Applied Biosystems), 2mM $MgCl_2$, 2% DMSO and 10% reaction buffer provided by the manufacturer. The amplification was performed using an initial denaturation step at 94°C for 2min, followed by 35 cycles of denaturation at 94°C for 1 min before annealing at 53°C for 2 min with extension done at 72°C for 1 min. The fina extension step was designated at 72°C for 10 min. All PCR products were electrophoresed on 1% Sea kem® GTG® agarose gels (FMC BioProducts, Rockland, ME, USA).

DNA sequencing and construction of plasmids for promoter analysis

The 546-bp PCR product from 28 Japanese Black cattle samples was cloned

into vector pT7Blue-T (Novagen, Madison, WI, USA) and sequenced on both strands using primers -21 M13 Forward, M13 Reverse or BPrP+83R and the ABI PRISM® Big Dye terminator cycle-sequencing ready reaction kit (Applied Biosystems). Products of the sequencing reaction were run on the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). The 516-bp fragment, including representative nucleotide substitutions, was cloned into vector pT7Blue-T after PCR amplification of the plasmids using BPrP-213-F (5'–AGCAATTTCAGGGAGTGATGAGCC-3') and BPrP+53-R (5'-TCAAGCTTCTGCTTCTGCGAGAGAGAAGACGC-3') before further subcloning into the luciferase reporter gene pGL3-Basic (Promega, Madison, WI, USA) (Fig. 1). Sequencing data-analysis was performed with DNASIS-MAC Ver.3 software (Hitachi, Yokohama, Japan). Gene sequences utilized in the comparative analysis were derived from original published DNA sequences of Genbank (AJ298878, Bos taurus). In the present study, the *PRNP* sequence of the Japanese Black was deposited in GenBank with the registration number AB250201, AB250202, AB250203, AB250204, AB250205, AB250206, and AB250207.

Cell culture

Neuroblastoma cells (N2a) were cultured in Eagle's minimum essential medium with non-essential amino acids (NEA), sodium pyruvate, and supplemented

with 10% fetal calf serum (FCS) at 37°C under 5% CO_2 for the luciferase assay.

Transient transfection luciferase assay

In transient transfection assays, cells were seeded in 24-well plates at 2 x 10^4 cells/well 24 h before transfection. Cells reaching 60-80% confluency was transfected with 2 μg/well for each test construct, using vector pRL-SV (2 μg/well). Luciferase activity of cell lysates prepared at 48 h post-transfection was measured as relative light units using a Fluoroscan Ascent FL (Labsystems, Franklin, MA, USA). The promoter-free vector Luc (pGL3-Basic) and the SV40 promoter containing vector Luc (pGL3-control, Promega) were transfected as negative and positive controls of promoter activity, respectively.

1.4. RESULTS

Deduced amino acid sequence of *PRNP* open reading frame from JB1~JB45 did not show any polymorphisms as compare to Holstein Friesian cattle (data not shown). In analyses of *PRNP*-promoter region polymorphisms in Japanese Black cattle breeds, genomic DNA was isolated from fat tissues of 45 Japanese Black cattle (JB1~JB45). To detect polymorphisms existing in Japanese black cattle population in Japan, a 546-bp fragment of the bovine *PRNP*-promoter region together with exon 1 was amplified using primers BPrP-463F and BPrP+83R for DNA sequencing (Fig. 1). The PCR products were electrophoresed on 1% agarose gels, and the expected 546-bp bands were amplified from the cattle genomic DNAs. The PCR products cloned into vector pT7Blue-T were sequenced on both strands. The sequencing results were compared with bovine *PRNP* (AJ298878). From the 28 Japanese Black cattle, 7 different kinds of polymorphic sites were found in the bovine *PRNP*-promoter region (Table 1). Of these 7 polymorphisms, 5 have not been reported previously and two previously described polymorphisms located at position -184A>G and -85T>G were confirmed in the Holstein breed as well (Hills et al., 2001; Sander et al., 2005). Among the newly identified variants, 2 polymorphisms were located in the Sp1-binding site of the bovine *PRNP*-promoter region (Fig. 2). The C→T and C→A nucleotide substitutions were within two Sp1-binding sites: one was located at position -6 and the other at position -47 (Fig. 2). Of the 28 Japanese Black cattle, 20 showed no nucleotide substitutions in the *PRNP*-promoter region (Fig. 2). The nucleotide substitutions were found at positions -141 and +17 of the *PRNP*-promoter region in 4 Japanese Black cattle, and the nucleotide substitutions were also found at other positions; i.e. (-184 and -85), (-184, -85 and -6), (-141 and +43) and (-141, -47 and +17) in the 4 Japanese Black

cattle. With reference to the allele frequencies of these polymorphisms (Table. 1), nucleotide substitutions at positions -6 and -47 located in the Sp1-binding sites were observed in each 1 sample. Nucleotide substitutions at positions -184, -141, -85, +17 and +43 were observed in 2, 6, 2, 5 and 1 samples, respectively. These results suggest that certain polymorphisms in bovine *PRNP*-promoter region were located in Japanese Black cattle breeds.

To examine activities of the bovine *PRNP*-promoter region, various reporter plasmids were constructed to analyze the promoter activity (Fig. 3). 516-bp (-463 to +53 bp) DNA fragments of the bovine *PRNP*-promoter region including nucleotide substitutions at (-184, -85), (-184, -85, -6), (-141), (-141, +17), and (-141, -47, +17) or no substitutions was cloned into a luciferase reporter vector pGL3-Basic. These reporter plasmids were transfected with vector pRL-SV40 into neuroblastoma cells N2a. The inserted DNA in the pGL-non plasmid did not contain nucleotide substitution in the *PRNP*-promoter region. These fragments were cloned into vector pGL3-Basic. The luciferase activities of cells transfected with several plasmids were compared to that of the pGL-non plasmid. The activity of pGL3-control transfected cells was taken as 100%. The plasmids pGL-184/-85 containing nucleotide substitutions at positions -184 and -85 showed 37.3% activity, which approximated to the luciferase activity comparable to the non-substituted sequences (36.3%) in N2a cells. However, the luciferase activity of the plasmid pGL-184/-85/-6 containing nucleotide substitutions at position -6 located in Sp1-binding site showed significantly attenuated activity approximating 31.4% of the luciferase activity in N2a cells. Plasmids pGL-141 and pGL-141/+17, containing nucleotide substitutions at positions -141 and (-141 and +17), manifested significantly suppressed activities approximating 27.4 and 26.9% of the luciferase activity in N2a cells. Furthermore, the luciferase activity of the plasmid pGL-141/-47/+17 showed a

decrease of ca. 24.2% of that in N2a cells (Fig. 3). The activities of the reporter plasmids pGL-141/+47/+17 and pGL-184/-85 were 10- and 16-fold more potent than the promoter-free reporter vector pGL3-Basic in N2a cells, respectively. Taken together, these results suggest that decrease in luciferase activity was induced by nucleotide substitution in Sp1-binding site or position -141 compared to the non-substituted *PRNP*-promoter region in N2a cells.

1.5. DISCUSSION

In this study, results of variabilities of the bovine *PRNP*-promoter region in Japanese Black cattle (consisted of three potential Sp1 binding sites) and the promoter activities in 28 Japanese Black cattle indicated 7 different kinds of polymorphic sites in the bovine *PRNP*-promoter region. Two kinds of polymorphic sites in German cattle (including the Holstein breed) have been in the same region (Sander et al., 2004). In comparison, variation within the bovine *PRNP*-promoter region was apparently greater in Japanese Black cattle breeds than Holstein breeds in the same region. All the identified variants harboured single-nucleotide polymorphisms (SNP), without any detectable insertions/deletions. There are three potential Sp1-binding sites within the bovine *PRNP*-promoter region (Fig. 3) at positions between (-11 and -6), (-27 and -22) and (-47 and -42) (Inoue et al., 1997). Among the newly identified variants, 2 kinds of polymorphic sites were located within the two Sp1-binding sites at positions between -47 and -6, respectively (Fig. 2). Previously published work suggests that transcription in G+C-rich promoters usually initiates at multiple-start sites between 40-100 nucleotides downstream of a Sp1 site (Suske et al., 1999). The assumed Sp1- binding sequences are probably GGGGCGGGGC and TGGGCGGGGC (Suske et al., 1999). Therefore, variations in the Sp1-binding site may influence the expression level. Promoter activities of the *PRNP* region from rat, bovine and mouse PrP^C genes have been reported previously. In the rat gene, deletions of box CCAAT (-66 to -41) and Sp1-binding sites resulted in a significant decrease of reporter gene activities, although these sites are opposite direction of consensus (Saeki et al., 1996). For the bovine gene, promoter activity has been identified within the 5'-flanking region from -88 and -30, which consisted of three putative Sp1-binding sites, which exist as opposite direction

(Inoue et al., 1997). There were C-to-A substitution at nucleotide -47 and C-to-T substitution at nucleotide -6. Statistical comparison between pGL-141/+17 and pGL-141/-47/+17 suggested that C-to-A substitution in the Sp1-binding sites at nucleotide -47 did not significantly affects *PRNP*-promoter activity (Fig. 3B). On the contrary, statistical comparison between pGL-184/-85 and pGL-184/-85/-6 suggested that C-to-T substitution in the SP1-binding sites at nucleotide -6 resulted in a significant decrease in luciferase activity compared with those of the non-substituted *PRNP*-promoter region (Fig. 3B). The transcription factor NEUROG1 was evaluated at bp position -85 of the bovine *PRNP*-promoter region by EMSA (electrophoretic mobility shift assays) (Sander et al., 2005). For the G/T polymorphism at position -85, no conclusive differences in the binding property of the respectively different alleles have been observed (Sander et al., 2005). These results suggest that variations in the Sp1-binding sites decreased the reporter gene activity, and Sp1-binding site polymorphisms could induce allele-specific expression levels. *PRNP*-promoter polymorphisms modulate *PRNP* expression and may influence the BSE incubation period and BSE susceptibility (Sander et al., 2005). Understanding the transcription of PrP^C expression is closely relevant to elucidation of the pathomechanisms leading to clinical symptoms in prion diseases.

In conclusion, there were certain polymorphisms in the bovine *PRNP*-promoter region of Japanese Black cattle, and Sp1-binding site polymorphisms could influence the promoter activity. Interestingly, these polymorphisms may be involved in the lower levels of *PRNP* expression. My study suggest that breeding cattle with such lower expression levels of *PRNP* may be a useful approach in reducing BSE risk.

Chapter 2

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

2.1. SUMMARY

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 bp 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 23bp Del/12bp Ins or 23bp Ins/12bp Ins showed lower promoter activity compared with other haplotypes (23bp Del/12bp Ins or 23bp Ins/12bp Del with -47C) or the wild-type haplotype (23bp Del/12bp 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.

2.2. INTRODUCTION

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 disease in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle (Prusiner, 1998). 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 (Prusiner et al., 1990; Bueler et al., 1993; Collinge et al., 1995). These findings are also supported by the fact that PrP-null mice that do not express PrP are completely resistant to infection with prions (Bueler et al., 1993) 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 (Bueler et al., 1993).

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 *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) (Bossers et al., 1996). 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.

2.3. 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 3 Japanese Black cows that were infected with BSE in Japan. Genomic DNA was isolated from the fat tissue samples by phenol/chloroform extraction.

DNA Amplification

Three parts of the bovine *PRNP* promoter region were amplified by polymerase chain reaction (PCR) (Table 2). 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) [7]. PCR was performed in a 50 μl reaction volume containing 200 ng DNA, 2.5 units 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 sec, 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 bp 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.[7].

DNA Sequencing and Construction of Plasmids for Promoter Analysis

The 864 bp, 165 bp, 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).

2.4. RESULTS

Analysis of the 12 bp and 23 bp Ins/Del polymorphisms of *PRNP* gene in Japanese Black cattle was performed and 44 samples were genotyped (Table 3). Representative electropherograms for the 12 bp and 23 bp indel polymorphisms are shown in Fig. 4. 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. 5A). 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 3). Similarly, the 23-bp deletion was homozygous in 47.7% of Japanese Black cattle, which was higher than the rate for the homozygous 23-bp insertion (9.1%) (Fig. 5 B, Table 3). The allele frequencies are shown Table 4. Constructs with a DelIns pattern were a very rare haplotype, and the InsDel pattern was not found in this study (Table 4). We also found an InsIns-Sp1 haplotype in this study (Table 4).

To examine the influence of the 12 bp and 23 bp indels and SNPs on gene expression, six reporter plasmids were constructed (Fig. 6A). DNA fragments, 1267 bp (-1634 to -1470 bp, -463 to +400 bp and +2389 to +2526 bp), of the bovine *PRNP* sequence including either the ins- or the del-alleles of both the 23 bp and 12 bp indel polymorphisms, the single nucleotide polymorphism (SNP) that was described in our previous study [8], and a partial region of exon 2 were cloned into a luciferase reporter

vector pGL3-basic. These reporter plasmids were transfected along with vector pRL-SV40 into neuroblastoma cells N2a and bovine fibroblast-like epithelial CKT-1 cells. All six *PRNP* constructs tested in the forward orientation exhibited promoter activity as indicated by the induction of luciferase reporter gene expression in both cell lines. The activity of pGL3-control-transfected cells was taken as 100%. The plasmid DelDel showed 175.6% activity, which was significantly higher level than the plasmids DelIns-Sp1 (128.3%) and InsIns-Sp1 (112.5%,) in CKT-1 cells (Fig. 6B). The activity from the plasmid DelDel was significantly higher than the plasmid DelIns-Sp1in N2a cells. There was a significant difference between the plasmids InsIns and InsIns-Sp1 in N2a cells but not in CKT-1 cells (Fig. 6B and C). However, the plasmid InsDel showed 162.9% activity, which was significantly higher than the plasmid InIn-Sp1 (112.5%) in CKT-1 cells (Fig. 6B), and a similar pattern (217.9% to 164.3% activity) was seen in N2a cells (Fig. 6C). The plasmid DelDel did not show a significantly higher level than the plasmid DelIns in either cell line (Fig. 6B, C). However, the plasmid DelDel showed a significantly higher level than plasmid DelIn-Sp1 in both cell lines (Fig. 6B, C).

To investigate the influence of the 12 bp and 23 bp indels and SNP on the binding activity of transcription factor Sp1 to the *PRNP* promoter, gel shift assays were conducted with nuclear extract from N2a cells. The experiment showed that the 23bp Del/12bpDel haplotype produced a strong, specific band shift (Fig. 7, lane 1). However,

the 23bp Del/12bp Ins with -47 haplotype produced only a weak band shift indicating that the 12-bp insertion and SNP could reduce the binding affinity of Sp1 to the *PRNP* promoter (Fig. 7, lane 3). Similarly, the 23bp Ins/12bpDel haplotype produced a stronger band shift than the 23bp Ins/12bp Ins with -47 haplotype (Fig. 7, lanes 6 and 5). In the absence of the N2a cell nuclear extract or DNA fragments, no complexes were observed (Fig. 7, lanes 7 and 8).

Taken together, these results suggest that concomitant substitution of the Sp1 binding sites upstream of exon 1 and the 12-bp insertion polymorphism in intron 1 showed lower expression levels than haplotypes with no substitution in the Sp1 binding site or the 12-bp deletion.

2.5. DISCUSSION

In this study, we examined the 23 bp and 12 bp indel polymorphisms in the *PRNP* promoter region from 44 Japanese Black cattle and the influence of 23 bp and 12 bp indel, and SNP polymorphisms on the promoter activity. These three variable sites, a 23 bp indel polymorphism containing the binding site for the transcription factor RP58, a 12 bp indel, and an SNP polymorphism containing a Sp1 binding site were described in European Friesian cattle (Inoue et al., 1997; Sander et al., 2005).

In a previous study, the 23-bp insertion was found to occur more frequently in healthy cattle than in BSE-infected cattle by Sander et al. (Sander et al., 2005). Recently, Geldermann et al. genotyped three polymorphic loci in introns 1 and 2 of *PRNP*, the 12 bp indel, R16, and R18, which showed significantly different distributions in BSE and control animals, whereas two further microsatellites located upstream of the 23 bp indel showed no significant differences (Geldermann et al., 2006). In our study using Japanese Black cattle, the 23-bp insertion has been found to have a lower allele frequency than the 23-bp deletion. However, we identified that there are 23-bp and 12-bp insertions and Sp1 substitution alleles in Japanese Black cattle breeds (Table 3). In this study this allele was shown to reduce promoter activity (Fig 3). The construct DelIns is a very rare haplotype, and InsDel was not found in this study. The InsDel

haplotype has not yet been found in any animals at all, neither in sequence variability studies in European Friesian cattle (Sander et al., 2004) nor in a quantitative RT-PCR study (Sander et al., 2005).

From the result of the luciferase assays, we deduced that concomitant substitutions of the Sp1 binding site in the upstream of exon 1 and the 12-bp insertion polymorphisms in intron 1, influenced the Sp1 interaction and reduced transcriptional activity. In gel shift assays, we observed that the substitution -47C→A and the 12-bp insertion in intron 1 reduced the intensity of the specifically retarded complex. Thus, the substitution -47C→A and the 12-bp insertion may exert indirect effects on the Sp1-dependent enhancement of transcription by disrupting the binding of other transcription regulators that might cooperate with Sp1 or bind directly to the promoter region. Previous studies have shown that multiple Sp1 sites within the same gene result in DNA looping when the Sp1 molecules bound to those sites interact with one another or with other proteins (Kim et al., 1999). This promotes interactions between promoters and distant regulatory elements and between different transcription regulators.

Transcription factor Sp1 is a sequence-specific transcription factor that binds to GC-rich sequence elements (GC boxes) in a wide variety of viral and cellular promoters (Dynan et al., 1983 (Cell 32); Dynan et al., 1983 (Cell 35); Dynan et al., 1985; Gidoni et al., 1985 (Nature 312); Gidoni et al., 1985 (Science 230)). Sp1 binding sites are usually

found within a few hundred base pairs of the transcriptional start site (Dynan et al., 1985). Furthermore, moving these binding sites away from the transcription start site resulted in a drastic decrease in promoter efficiency (Moreau et al., 1981). For the bovine *PRNP* gene, promoter activity has been identified within the 5'-flanking region from -88 and -30, which consists of 3 putative Sp1 binding sites in the opposite direction (Inoude et al., 1997). In a previous study, we reported that there is a polymorphic site located within the Sp1 binding sites at position -47 (Nakamura et al., 2007). The 12-bp indel polymorphism in intron 1 was included in the analysis as it has been shown previously that intron 1 elements are necessary for bovine *PRNP* promoter activity (Inoue et al., 1997). Distally and proximally bound Sp1 can stimulate transcription synergistically (Courey et al., 1989). Sp1-Sp1 interactions may play an important role in modulating the promoter activity (Courey et al., 1989). Our reporter gene assays revealed that the combination between the 12-bp insertion and the substitution in the Sp1 binding site decreased the expression level more than the 12-bp deletion with no substitution irrespective of the presence of the 23-bp insertion or deletion. Mutation inactivation of either the Sp1 or the E2F site almost completely abolishes promoter activity (Karlseder et al., 1996). Therefore, substitution in the Sp1 binding site may negatively affect the Sp1-Sp1 interaction and reduce transcriptional activity.

Polymorphisms in the 5'-flanking region of the *PRNP* gene may result in reduced expression, and be associated with supressing BSE progression. Identification of the regulatory sequence of the 5'-flanking region of *PRNP* gene may be useful for understanding the pathophysiology of prion diseases.

Chapter 3

Investigation of PrP overexpression to the bovine *PRNP* promoter region

3.1. SUMMARY

The novel neurologic syndromes in transgenic mice with high levels of PrP^C encoded by wild-type transgenes add to the growing spectrum of prion diseases. There are hypothesis that overexpression of PrP^C could result from a variety of genetic or epigenetic insults: promoter or enhancer mutations, mutations in sequences controlling mRNA stability or translation, or physiological conditions that stimulate PrP gene transcription. To investigate the PrP overexpression to the bovine *PRNP* promoter, bovine *PRNP* promoter luciferase vector and bovine PrP expression vector were transfected into N2a cells. Activity of some reporter plasmid was inhibited by Overexpression PrP.

3.2. INTRODUCTION

The spongiform encephalopathies (prion diseases) such as Creutzfeldt-Jakob disease and Gerstmann-Straussler Scheinker syndrome in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in cattle are fatal neurodegenerative disorders with pathologies including neuronal cell loss, vacuolation, astrocytosis and amyloid plaques (Prusiner, 1998). The expression of a glycosylphosphatidylinositol (GPI)-anchored plasma membrane cellular prion protein PrP^{C} is required for the propagation of the prion (Bueler et al., 1993). Prion infection is attributed to entry of a proteinaceous particle into normal brain, leading to accumulation of an abnormally folded form of the prion protein (PrP^{Sc}) as a consequence of conformational conversion from the endogenous PrP^{C} (Prusiner, 1998).

The prion gene plays a central role in the TSEs. PrP^{C} is constitutively expressed on the cell surface of neural and nonneuronal cells (Bendheim et al., 1992). An expression of PrP^{C}-deficient cells undergoing apoptosis exhibited repeatedly antiapoptotic effects. However, overexpression of PrP^{C} is associated with degeneration of skeletal muscle, peripheral nerves, and the central nervous system (Westaway et al., 1994). Therefore, the presence/absence and/or the level of PrP^{C} expression seem to be critical for the fluctuation between PrP^{C}'s pro- and antiapoptotic properties.

To investigate the PrP overexpression to the bovine *PRNP* promoter, bovine

PRNP promoter luciferase vector and bovine PrP expression vector were transfected into N2a cells. Activity of some reporter plasmid was inhibited by Overexpression PrP.

3.3. MATERIALS AND METHODS

Construction of plasmids for protein expression

The open reading frame (ORF) of bovine *Prnp* were amplified from Japanese black cattle genomic DNA (JB2, JB3, JB25) by polymerase chain reaction (PCR) using the following primers.

(a) sense primer 5′-AGctcgagATGGTGAAAAGCCACATAGGCAGT-3′

(b) antisense primer 5′-TCgcggccGCCTATCCTACTATGAGAAAAATGAG-3′,

The low-case letters indicate the restriction sequences of *Xho* I and *Not* I, respectively. PCR products were cloned into a pT7BlueT-vector, followed by DNA sequencing using an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., USA) and compared with the database sequences of bovine *PPNP* (Gene Bank: AJ298878). Plasmid containing the ORF BoPrP was subsequently cloned into the vector pEF-BOS (Mizushima et al., 1990) to produce pEF-boPrP. The DNA sequences of the ORF of bovine *PRNP* in the pEF-BOS vectors were verified by sequencing using an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., USA).

Cell culture and transfection

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.

To investigate absolute transfection efficiency, green fluorescent protein (GFP) expression vector were transfected into N2a cells.

For the promoter assays, 5×10^4 N2a cells were seeded 48 h before transfection into 24-well plates. Cells reaching 60-80% confluency were co-transfected with 500 ng of bovine *PRNP* promoter luciferase vector which were described before (Xue et al., 2008), 2 μ g of bovine PrP expression vector and empty vector, and 5 ng of pRL-SV internal control plasmid. The transfections were carried out using Lipofectamine^{TM} LTX and PLUS^{TM} reagents according to the manufacturer's protocol (Invitrogen).

Recombinant bovine PrP and HRP-labeling of mAb 1D12

The recombinant bovine PrP (25-241) (Alicon) used in this study was provided from the Japan Red Cross Society. The mAb 1D12 was labeled with HRP using the Peroxidase-labeling Kit-SH (Dojindo) according to the manufacturer's instructions.

Western blot assay

Cell pellets were suspended in radio-immunoprecipitation assay (RIPA) buffer composed of 10 mM Tris-HCl (pH 7.4) containing 1% sodium deoxycholate, 1%

Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS) and 0.15 M sodium chloride supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF) and lysed in 2 X SDS gel-loading buffer [90 mM Tris/HCl (pH 6.8), 10% mercaptoethanol, 2% SDS, 0.02% bromophenol blue, 20% glycerol]. The samples were boiled for 5 min before an equal quantity of protein (70 μ g for C6, untransfected N2a and pEF-boPrP transfected N2a) was subjected to electrophoresis on SDS/12% polyacrylamide gel. Proteins electrically transferred onto polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham-Pharmacia Biotech, Piscataway, NJ) were treated by BLOCK-ACE (Dainippon pharmaceutical, Osaka, Japan) at 4°C overnight. Membranes were then incubated in PBS containing 0.1% Tween-20 (PBS-T) and 10% BLOCK-ACE for 1 h at room temperature with one of the following antibodies: T2 monoclonal anti-PrP, SAF83 and 6H4 (Prionics, Zürich, Switzerland) (Korth et al., 1997). As a loading control antibodies α-tubulin (Sigma) was used at /12000 dilution. After washing with PBS-T, the membrane was incubated in secondary antibody: peroxidase-labeled anti-mouse IgG (diluted 1:5000 in PBS-T) (Amersham Biosciences) for 1 h at room temperature. After three washes in PBS-T, the probed proteins were detected using the chemiluminescent substrate (ECL^{TM} Detection Reagents; Amersham Biosciences) by a lumino image analyzer (LAS-3000 mini; Fujifilm).

Sandwich ELISA

Microtiter plates (Nunc-Immuno^{TM} Modules; Nunc) were coated with 100 μ l of mAb 1D12 (1 μ g/ml) in 0.1 M carbonate buffer (pH 9.5) overnight at 4℃ and washed with PBS-T three times. The coated plates were blocked 200 μ l of Block Ace (diluted 1:4 in PBS-T) for 1h at room temperature and subsequently rinsed with PBS-T three times. 100 μ l of diluted samples in PBS were added to the wells. The plates were incubated for 1h at room temperature. The plates were washed with PBS-T five times before adding 100 μ l of HRP-labeled anti-PrP mAb 1D12 (0.5 μ g/ml) in PBS-T to the wells. After washing with PBS-T, 100 μ l of o-phenylenediamine (Sigma) solution was dispensed into each well. After incubating 30 min in the dark box, 20 μ l of 6 N H_2SO_4 was added to the wells and the absorbance was read at 490 nm on a Microplate reader (Bio-Rad).

Luciferase assay

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.

3.4. RESULTS

To examine absolute transfection efficiency, N2a cells were transfected with green fluorescent protein (GFP) expression vector (Fig. 9). 48 hours post transfection, the cells were analyzed by fluorescence microscopy. Approximately 80% of transfection efficiency was observed.

After identification of absolute transfection efficiency, 2.5µg, 3.75µg, 5µg and 10µg of pEF-boPrP (Fig. 8) and empty vector pEF-BOS were transfected into N2a cells. 48 hours post transfection, cell lysates were then analysed by western blotting (Fig. 10). In pEF-boPrP transfected N2a cells, an increase in PrP expression was visible by comparison of the signal intensity (Fig. 10, lane 3) with the untrasfected state. The level of PrP expression was performed by sandwich ELISA. The sandwich ELISA was carried out with combination of T2 as a capture mAb and 1D12-HRP as a detection mAb. The linear standard curve of recombinant bovine PrP is shown in Figure 11a. The expression level of PrP in N2a after transfection with 5µg, 10µg of pEF-boPrP was higher than that untransfected or transfection with empty vector cells (Fig. 11 b). These results suggest that pEF-boPrP could express bovine PrP in N2a cells efficiently.

To investigate PrP overexpression to the bovine *PRNP* promoter region, bovine *PRNP* promoter luciferase vector, bovine PrP expression vector pEF-boPrP, and

pRL-SV internal control plasmid were co-transfected into N2a cells. All constructs tested in the forward orientation exhibited promoter activity as indicated by the induction of luciferase reporter gene expression in the N2a cells. The activity of pGL3-control-transfected cells was taken as 100%. The relative activities of six reporter plasmids which co-transfected with pEF-BOS vector and pEF-boPrP, respectively, were all lower than transfected reporter plasmids only. Because pEF-BOS also decreased the reporter gene activities, thus reporter activity of co-transfection of pEF-boPrP have to compare with the co-transfection of empty vector. The activity of plasmid DelDel co-transfected with pEF-boPrP showed lower than pEF-BOS. However, there were no differences in other plasmid.

3.5. DISCUSSION

The posttranslational conformational change of PrP^{C} into PrP^{Sc} is the fundamental process underlying the pathogenesis of prion deseases (Prusiner et al., 1998). Many concurrent repots have suggested that PrP^{C} may play a role in neuronal survival or death. The removal of serum from cells in culture causes apoptosis in PrP^{C} -deleted cells but not in wild-type cells (Kuwahara et al., 1999). On the other hand, misfolded secretory proteins (Kopito et al., 1997), it is recognized in the ER and subject to retrograde transport to the cytoplasm and degradation by the proteasome (Jin et al., 2000; Ma et al., 2001; Yedidia et al.,2001)

To investigate PrP overexpression to the bovine *PRNP* promoter region, bovine *PRNP* promoter luciferase vector, bovine PrP expression vector pEF-boPrP, and pRL-SV internal control plasmid were co-transfected into N2a cells. The pEF-boPrP vector is a powerful mammalian expression vector, which has human EF-1 α chromosomal gene.

In this study, related activity of DelDel was inhibited by the overexpression of PrP. The plasmid DelDel constructed before, which contains the deletion of 23bp in upstream of *PRNP* promoter and 12bp deletion in intron 1(Xue et al., 2008). A 23 bp indel polymorphism containing the binding site for the transcription factor RP58, a 12

bp indel polymorphism containing a Sp1 binding site were described in European Friesian cattle (Inoue et al., 1997; Sander et al., 2005). This may be pDelDel element have response element to the PrP stimulate. However, a promoter activity of pGL3-control vector was no change by overexpression of PrP. The pGL3-control promoter is SV40, may be overexpression of PrP cannot suppress SV40 promoter. The overexpression of PrP can suppress *PRNP* promoter, but cannot suppress SV40 promoter.

GENERAL CONCLUSION

PRNP-promoter polymorphisms modulate *PRNP* expression and may influence the BSE incubation period and BSE susceptibility (Sander et al., 2005). Understanding the transcription of PrP^C expression is closely relevant to elucidation of the pathomechanisms leading to clinical symptoms in prion diseases. Therefore, the main purpose of this study were to investigate (1) *PRNP*-promoter region polymorphisms in Japanese Black cattle breeds and it promoter activity, (2) the relationship between transcription factor which binding bovine *PRNP* promoter region, (3) overexpression of PrP to the bovine *PRNP* promoter region.

In CHAPTER 1, novel single nucleotide polymorphisms (SNPs) located in the 5' flanking region of Bovine *PRNP* affecting its expression were demonstrated in Japanese Black cattle.

In CHAPTER 2, the 12 bp and an additional polymorphism (-47C→A) in the Sp1 binding site coordinately affect the promoter activity were investigated. 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.

In CHAPTER 3, the overexpression of PrP to the bovine *PRNP* promoter regions were investigated.

Taken together, bovine *PRNP* promoter region plays important roles in BSE.

And polymorphisms in *PRNP* promoter may be involved in the lower levels of *PRNP* expression. Our studies suggest that breeding cattle with such lower expression levels of *PRNP* may be a useful approach in reducing BSE risk.

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TABLES

Table 1. Sequence variants of the bovine prion protein gene (*PRNP*)-promoter region in Japanese Black cattle

Position[a]	Variant	GenBank Accession number	Allele number of the variant[b]
-184[c]	A>G	AJ298878	2
-141	T>C	This study	6
-85[d]	T>G	BN000291	2
-47	C>A	This study	1
-6	C>T	This study	1
+17	C>T	This study	5
+43	C>T	This study	1

[a] The position of the transcription initiation site is +1.

[b] Allele number in the 28 sequenced samples.

[c] This polymorphism has been documented in Hills et al. (Hills et al., 2001)

[d] This polymorphism has been documented in Sander et al. (Sander et al., 2005)

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FIGURE LEGENDS

Fig. 1. Organization of the bovine prion protein gene (*PRNP*).

The hatched and closed boxes denote the three exons of the *PRNP*. The protein-coding region in exon 3 (closed box), and exons 1 and 2, and the 5' and 3' non-coding regions in exon 3 (hatched boxes) are indicated accordingly. Two primers, BPrP-463F and BPrP+53R or BPrP-463-F and BPrP+83-R (arrows), were used to amplify the 516-bp or 546-bp fragments.

Fig. 2. Nucleotide sequences of the 5'-flanking region and exon 1.

The transcription-initiation site labelled as +1 is indicated by a vertical arrow. The three putative Sp1-binding sites (open boxes) and exon1 (grey boxes) are indicated accordingly. The nucleotide substitutions (blue boxes) are indicated under the sequence AJ298878, and the sequence positions are indicated at the right.

Fig. 3. Schematic representation of the reporter gene constructs.

(A) Six reporter gene were constructed (pGL-non, pGL-184/-85, pGL-184/-85/-6, pGL-141, pGL-141/+17, pGL-141/+47/+17). The pGL3-Basic was the promoter free vector control. The arrow denotes the position of the reported transcriptional start site (+1) of *PRNP* promoter region. The luciferase gene (dotted box) and exon1 (open box)

are indicated accordingly. Seven nucleotide substitutions are indicated above the reporter gene constructs. The allele frequency of six haplotypes are indicated on the right. (B) Luciferase activity was measured by luminometry of cell lysates prepared at 48 h post-transfection. Relative luciferase activities (mean ± SEM) for triplicate experiments were compared with the pGL3-control plasmid (100%). Differences where $p<0.05$ (*) and <0.01 (**) were considered statistically significant when compared with the pGL-non plasmid with the non-repeated measures ANOVA followed by the Bonfferoni analysis. Difference between pGL-184/-85 and pGL-184/-85/-6 was statistically significant [$p<0.05$ (c)], whereas difference between pGL-141/+17 and pGL-141/-47/+17 was not significant (N.S.) with the non-repeated measures ANOVA followed by the SNK analysis.

Fig. 4. Identification of the G/T transition, 12-bp indel and 23-bp indel polymorphisms in the bovine *PRNP* promoter region.

(A) Electropherogram showing the base substitution at the position -47 of the bovine *PRNP* promoter. (B) Schematic model of the bovine *PRNP* promoter. The relative position of the SNP is indicated by arrow.

Fig. 5. Genotyping of insertion (Ins) or deletion (Del) polymorphisms in the promoter region of bovine *PRNP*.

The PCR products using the following primers were subjected to acrylamide gel electrophoresis. Homozygous genotypes (+/+) and heterozygous (+/-) genotypes of the 12 bp Ins or wild type genotype (-/-) in intron 1 of the bovine *PRNP* gene was assessed using primer pair PRNP49686_F and PRNP49777_R (A), while those of the 23 bp Ins polymorphism in the 5'-flanking region of the bovine *PRNP* gene were assessed using primer pair PRNP47784_F and PRNP47883_R (B).

Fig. 6. Influence of the insertion (Ins) and deletion (Del) polymorphisms on transcriptional activities of bovine *PRNP*

(A) A map of the portion of the bovine *PRNP* containing the 5'-flanking region and exons 1 and 2 is shown in the top line. Six reporter genes were constructed (DelDel, DelIns-Sp1, DelIns, InsDel, InsIns-Sp1, and InsIns). pGL3-basic was the promoter-free vector control. Dotted box= Luciferase gene; black boxes= exon 1 and exon 2, which include arrows denoting the position of the reported transcription start site (+1) of the *PRNP* promoter region. The 23 bp indel, 12 bp indel, and SNP regions are indicated above the reporter gene constructs. (B) Luciferase activity was measured by luminometry of cell lysates prepared 48 h after transfection. Relative luciferase

activities (Mean ± SEM) for 3 replicate experiments were compared with the pGL3-control plasmid (1.0). (B) Transfection in CKT-1 cells. (C) Transfection in N2a cells. (*P<0.05 and **P<0.01, Non-repeated Measures ANOVA followed with Student-Newman-Keuls test).

Fig. 7. Gel shift assay

Gel shift analysis of Sp1 binding to the *PRNP* promoter sequence. The arrow indicates the shifted band. Negative controls lacked nuclear extract or DNA fragments.

Fig. 8. Construction of expression vector and amplification of bovine PrP gene

TOP: Map of expression vector

BOTTOM: PCR products of the open reading frame (ORF) of bovine *Prnp*

Samples from Japanese black cattle genomic DNA (JB2, JB3, JB25).

Fig. 9. Investigation of absolute transfection efficiency by green fluorescent protein (GFP).

(A, B) 48 hours post transfection the Neuro-2a cells were analyzed by fluorescence microscopy. The high transfection efficiency of GFP was observed. (C, D) Negative control. Scale bar is 50 μm.

Fig. 10. N2a cells transfectd with pEF-boPrP

The constructed Bovine PrP expression vector, pEF-boPrP were transfected into N2a cells. At 48 hr post transfection, cell lysates were prepared. C6 was taken as a positive control. A) PrP were detected with T2 (1 μ g/ml), B) SAF 83(1 μ g/ml), and C) 6H4 (1 μ g/ml). D) α -tubulin was taken as internal control.

Fig. 11. Bovine PrP detection in N2a cells by the sandwich ELISA

a) ELISA standard curve generated from recombinant bovine PrP.

b) Transfected bovine PrP in N2a cells was detected by the sandwich ELISA with T2 as a capture mAb and 1D12-HRP as a detection mAb. Each data point is the mean of three determinations. N2a only: un-transfection N2a-EM-2.5 μ g: 2.5 μ g of pEF-BOS empty vector was transfected into N2a cells. N2a-PrP-2.5 μ g: 2.5 μ g of pEF-boPrP plasmid was transfected into N2a cells.

Fig. 12. Bovine *PRNP* promoter activity in N2a cells

Luciferase activity was measured by luminometry of cell lysates prepared 48 h after transfection. Relative luciferase activities (Mean ± S.D.) for 3 replicate experiments were compared with the pGL3-control plasmid (1.0).

Fig. 1

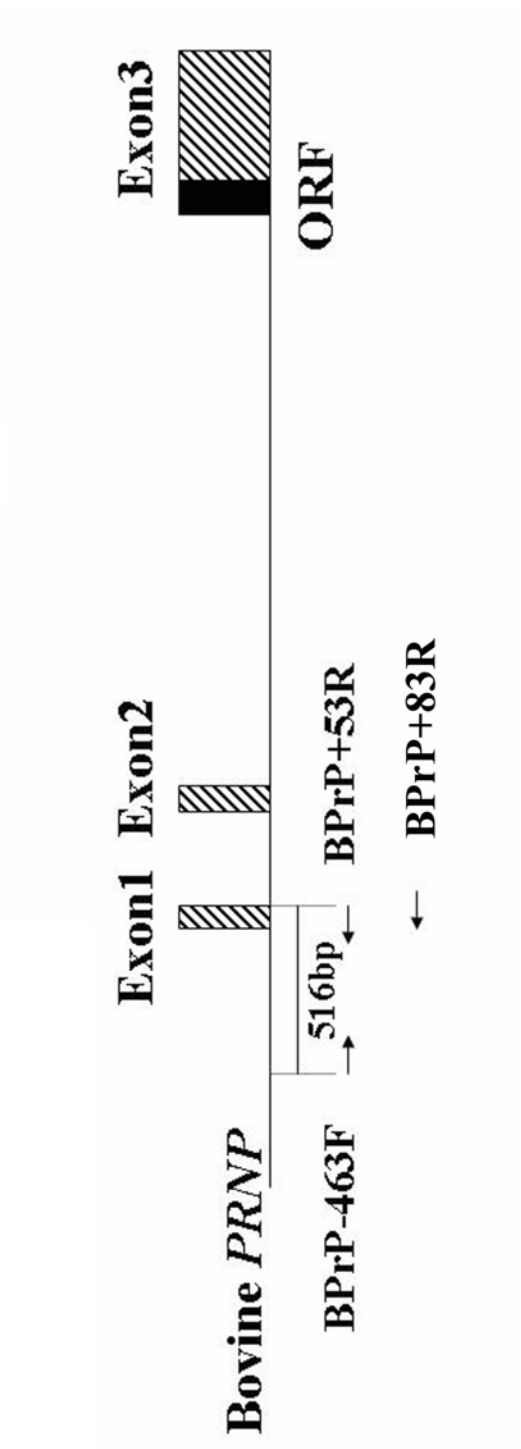

Exon3
ORF
Exon1
Exon2
Bovine PRNP
516bp
BPrP-463F
BPrP+53R
BPrP+83R

Fig. 2

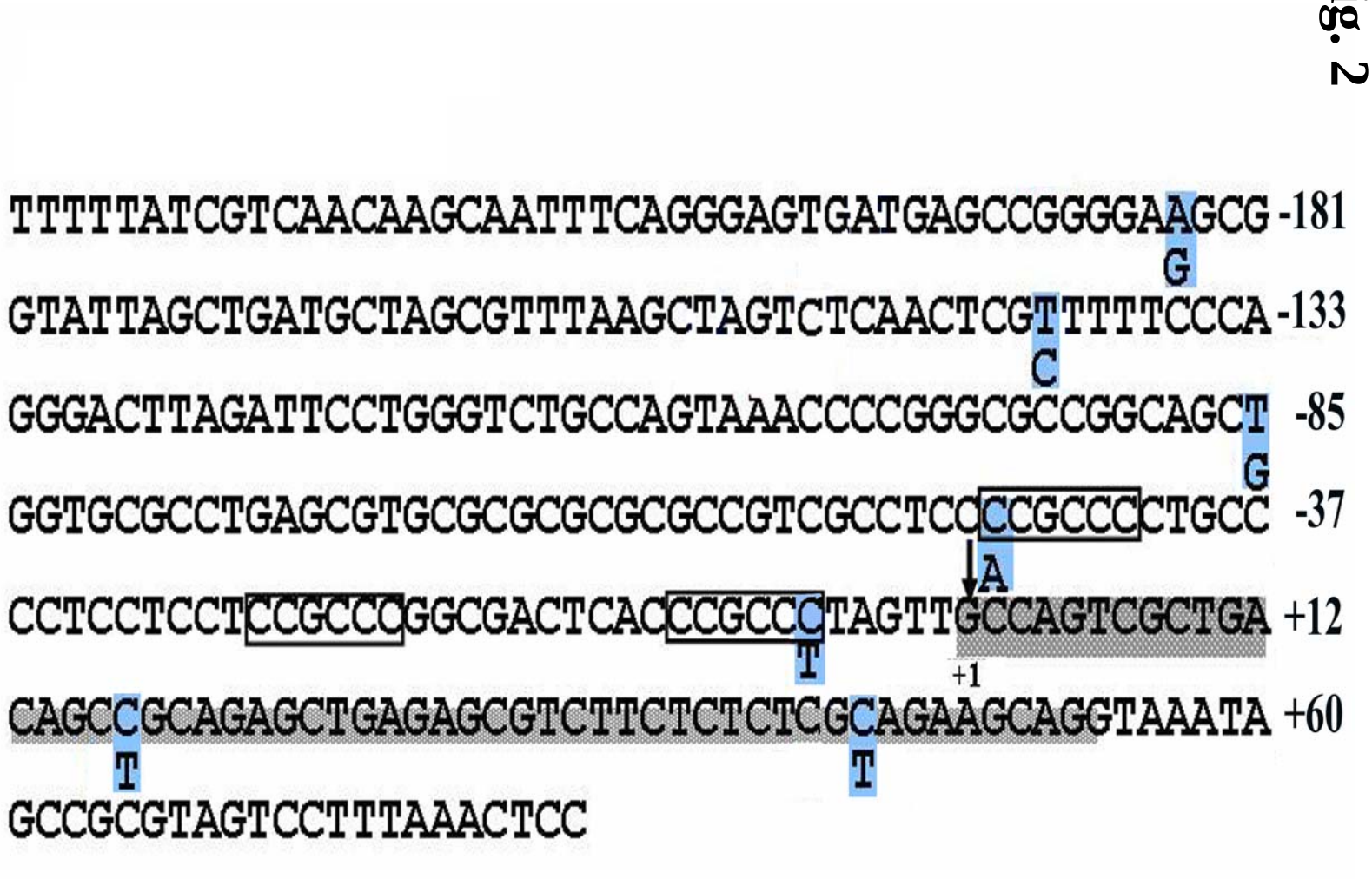

TTTTTATCGTCAACAAGCAATTTCAGGGAGTGATGAGCCGGGGAAGCG -181
G
GTATTAGCTGATGCTAGCGTTTAAGCTAGTCTCAACTCGTTTTTCCCA -133
C
GGGACTTAGATTCCTGGGTCTGCCAGTAAACCCCGGGCGCCGGCAGCT -85
G
GGTGCGCCTGAGCGTGCGCGCGCGCCGTCGCCTCCCCGCCCCTGCC -37
A
CCTCCTCCTCCGCCCGGCGACTCACCCGCCCTAGTTGCCAGTCGCTGA +12
T
+1
CAGCCGCAGAGCTGAGAGCGTCTTCTCTCTCGCAGAAGCAGGTAAATA +60
T
T
GCCGCGTAGTCCTTTAAACTCC

Fig. 3

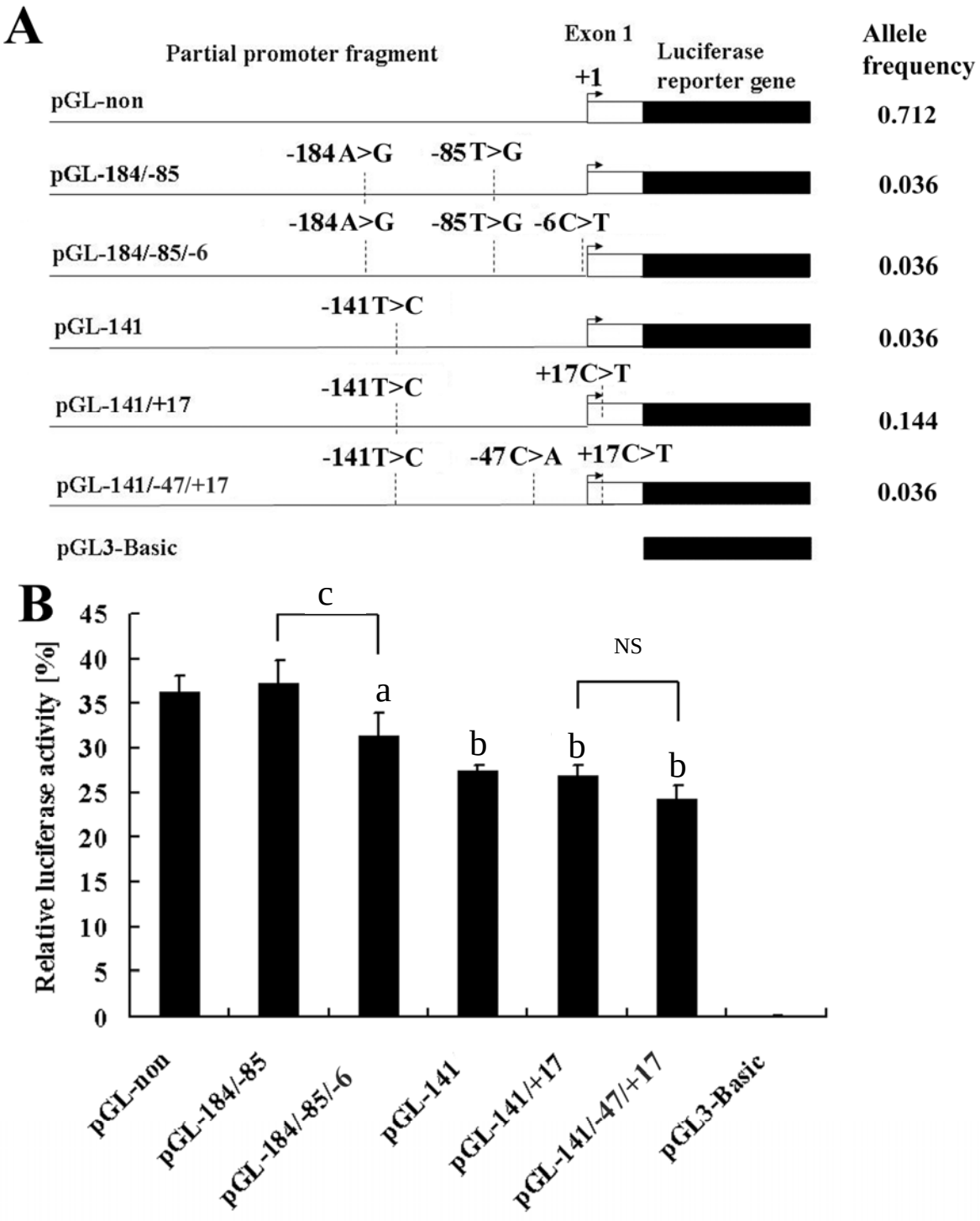
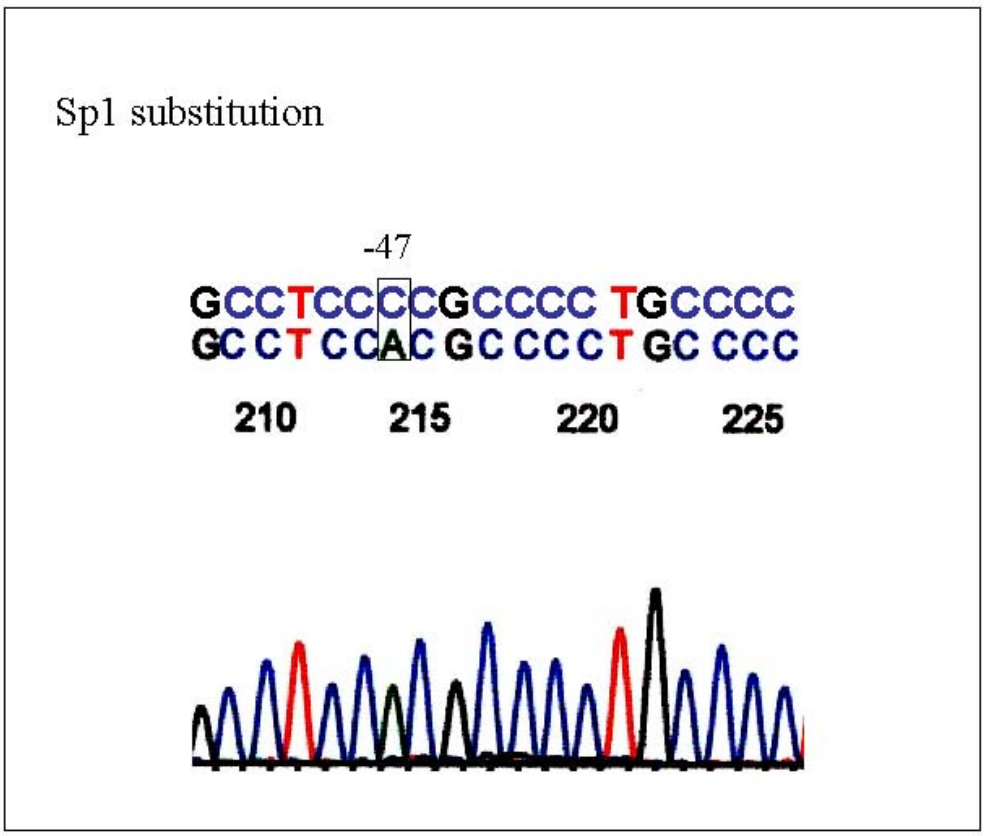
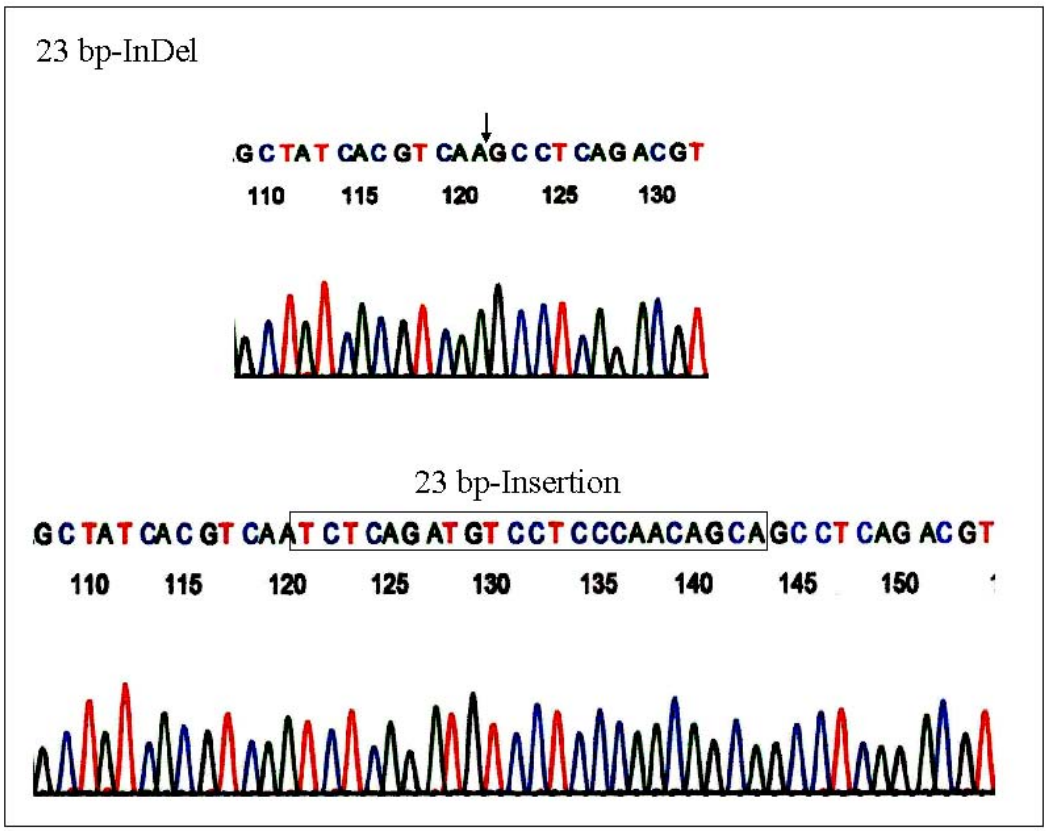

A
Partial promoter fragment
Exon 1
+1
Luciferase reporter gene
Allele frequency
pGL-non
0.712
-184A>G
-85T>G
pGL-184/-85
0.036
-184A>G
-85T>G
-6C>T
pGL-184/-85/-6
0.036
-141T>C
pGL-141
0.036
+17C>T
-141T>C
pGL-141/+17
0.144
-141T>C
-47C>A
+17C>T
pGL-141/-47/+17
0.036
pGL3-Basic
B
Relative luciferase activity [%]
45
40
35
30
25
20
15
10
5
0
c
NS
a
b
b
b
pGL-non
pGL-184/-85
pGL-184/-85/-6
pGL-141
pGL-141/+17
pGL-141/-47/+17
pGL3-Basic

Fig. 4

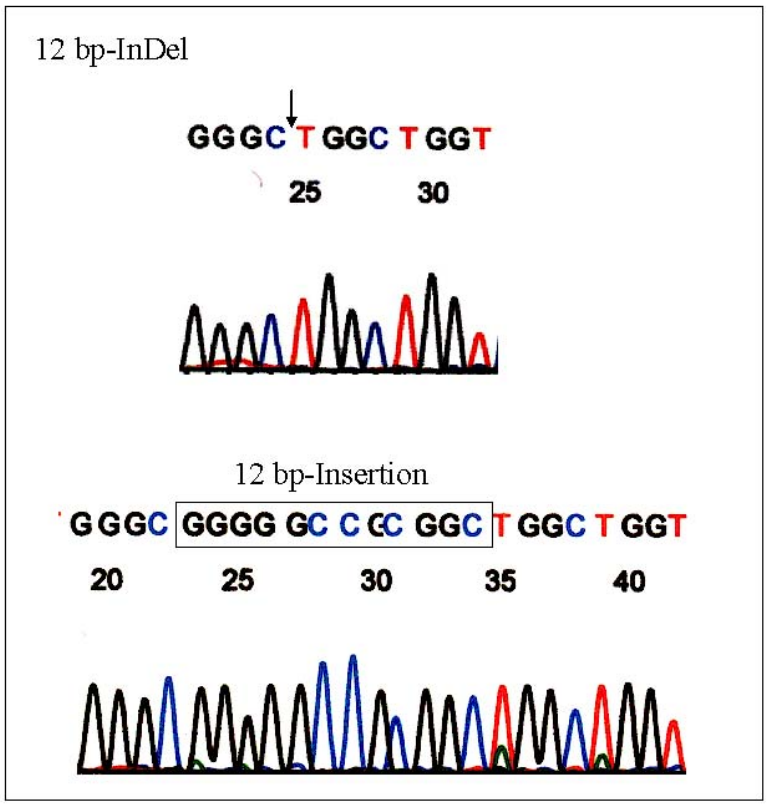
A-1


Sp1 substitution
-47
GCCTCCCCGCCCC TGCCCC
GC CT CCAC GC CCCT GC CCC
210
215
220
225

A-2


23 bp-InDel
GCTATCACGTCAAGCCTCAGACGT
110 115 120 125 130
23 bp-Insertion
GCTATCACGTCAATCTCAGATGTCCTCCCAACAGCAGCCTCAGACGT
110 115 120 125 130 135 140 145 150

A-3


12 bp-InDel
GGGCTGGCTGGT
25
30
12 bp-Insertion
GGGCGGGGGCCCCGGCTGGCTGGT
20
25
30
35
40

B

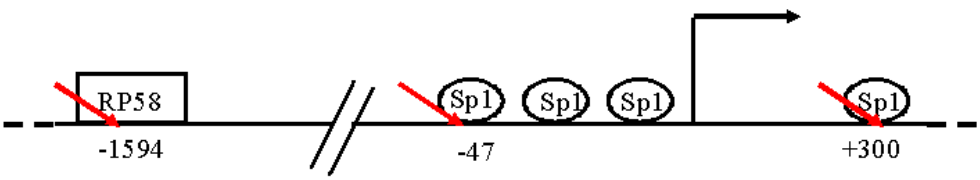

RP58
Sp1
Sp1
Sp1
Sp1
-1594
-47
+300

Fig. 5

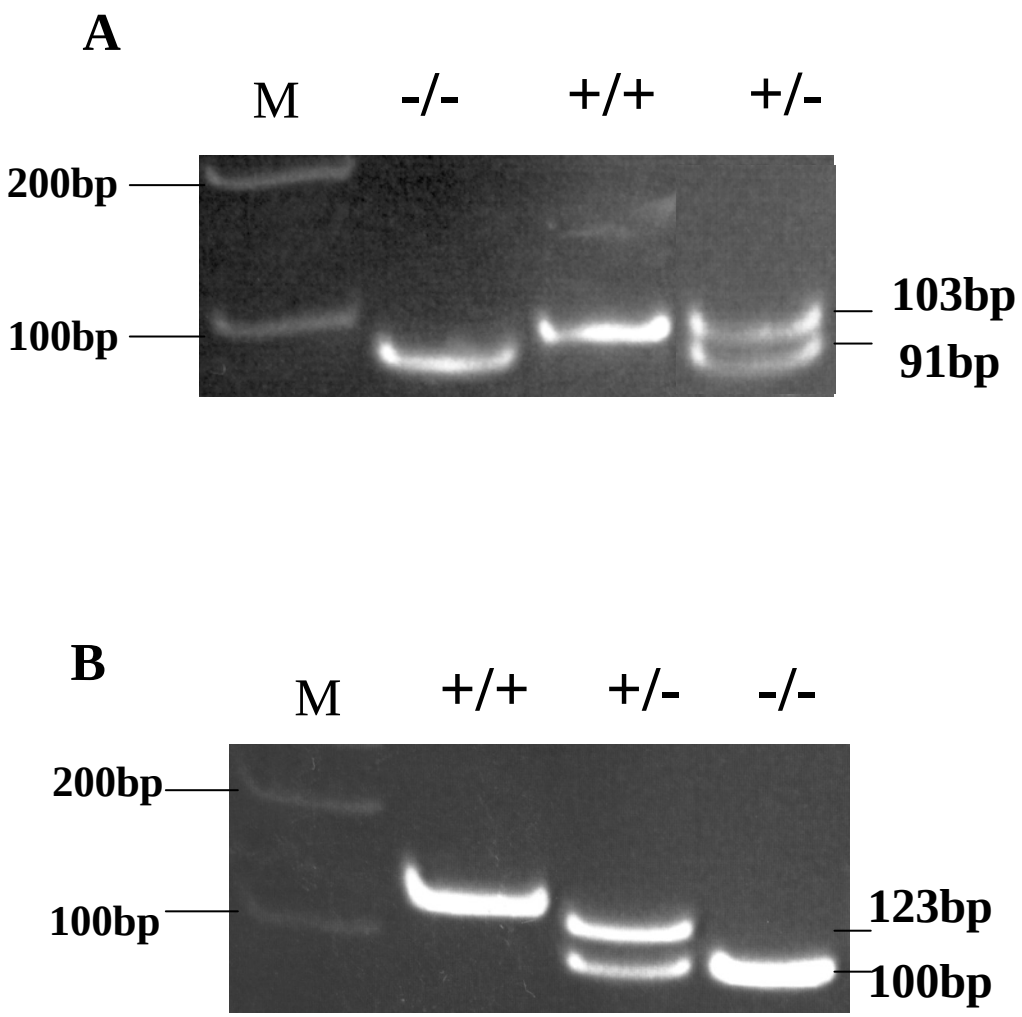


Fig. 6

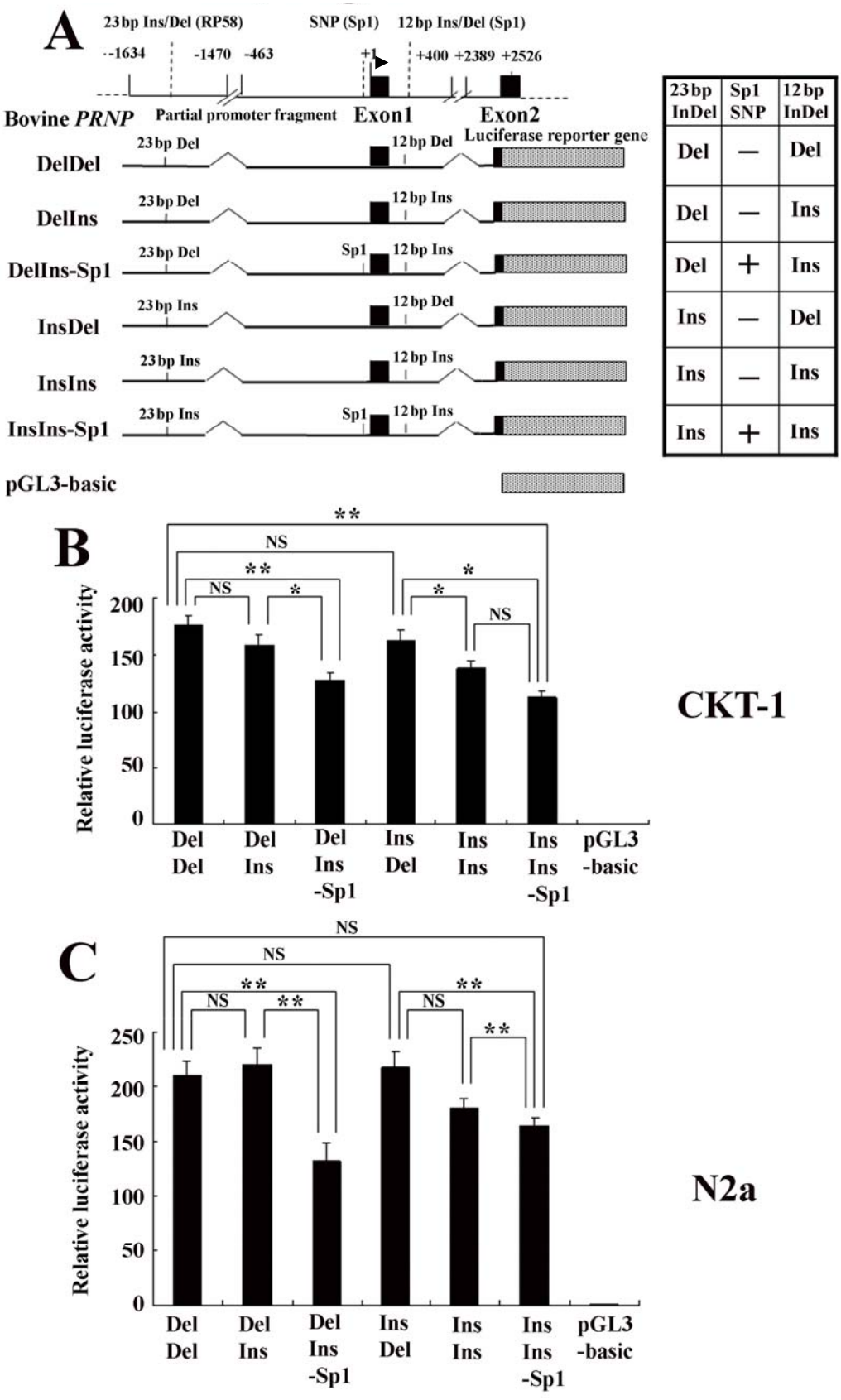

A
23bp Ins/Del (RP58)
SNP (Sp1)
12bp Ins/Del (Sp1)
--1634
-1470
-463
+1
+400
+2389
+2526
Bovine PRNP
Partial promoter fragment
Exon1
Exon2
Luciferase reporter gene
DelDel
DelIns
DelIns-Sp1
InsDel
InsIns
InsIns-Sp1
23bp Del
12bp Del
12bp Ins
23bp Ins
Sp1
pGL3-basic
23bp InDel
Sp1 SNP
12bp InDel
Del — Del
Del — Ins
Del + Ins
Ins — Del
Ins — Ins
Ins + Ins
B
CKT-1
Relative luciferase activity
0
50
100
150
200
NS
**
*
Del Del
Del Ins
Del Ins -Sp1
Ins Del
Ins Ins
Ins Ins -Sp1
pGL3 -basic
C
N2a
Relative luciferase activity
0
50
100
150
200
250
NS
**
*

Fig. 7

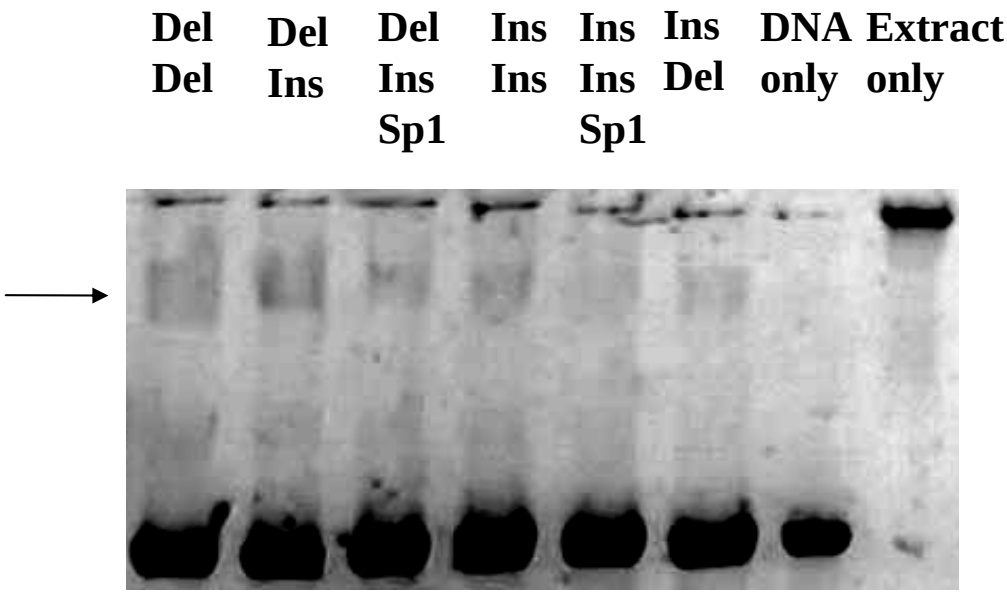
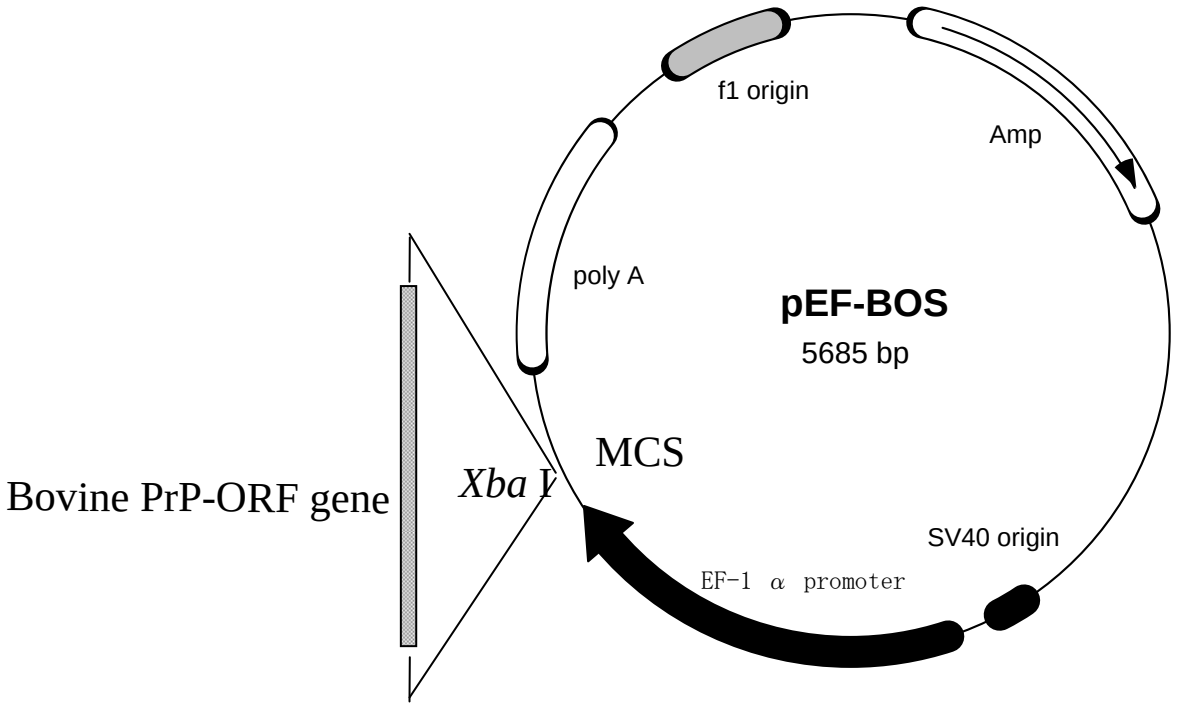

Del Del
Del Ins
Del Ins Sp1
Ins Ins
Ins Ins Sp1
Ins Del
DNA only
Extract only

Fig. 8 Construction of pEF-bovine PrP



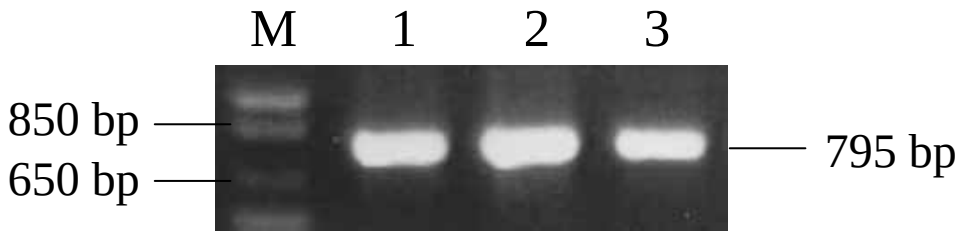


Fig. 9

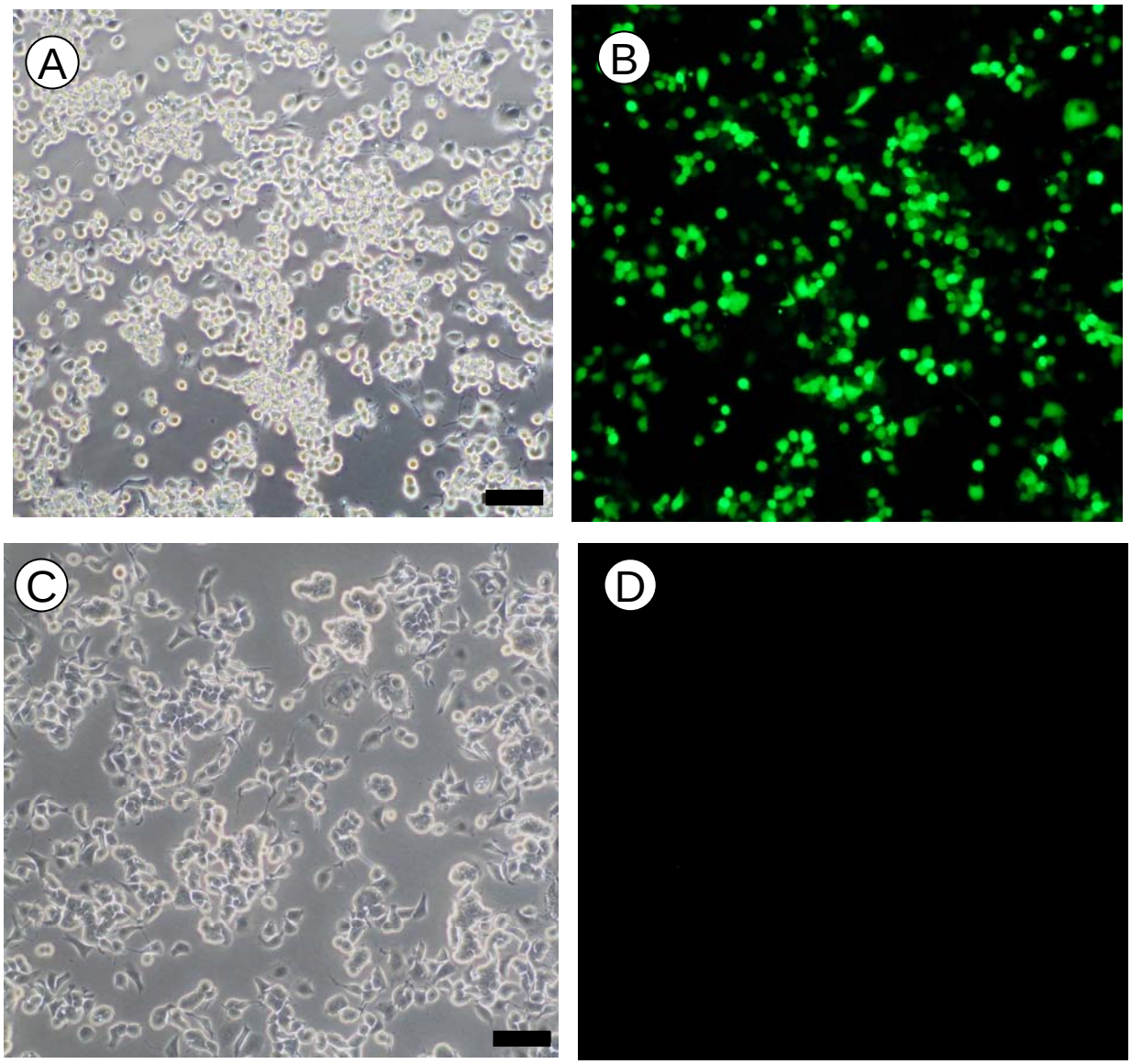


Fig. 10

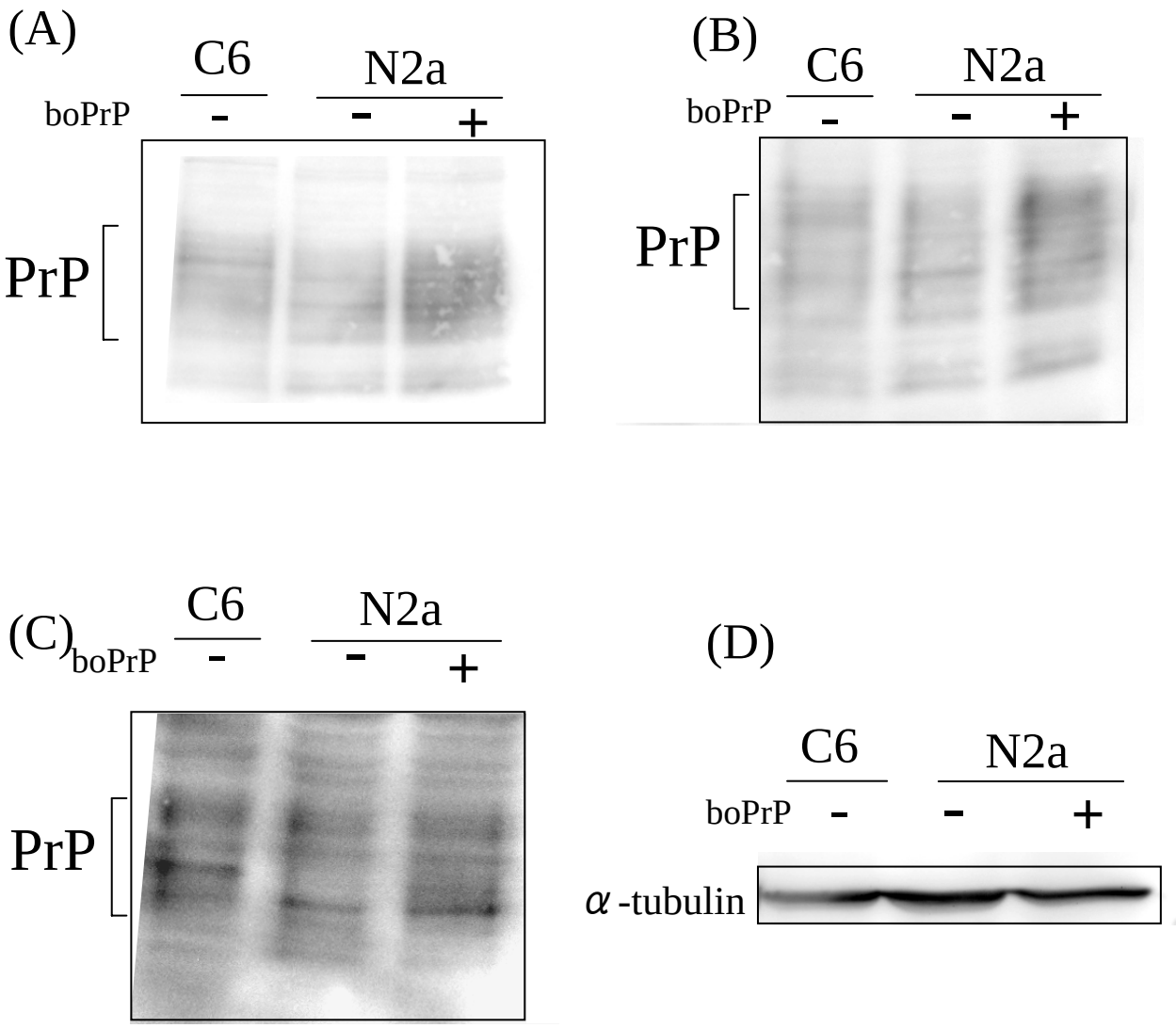
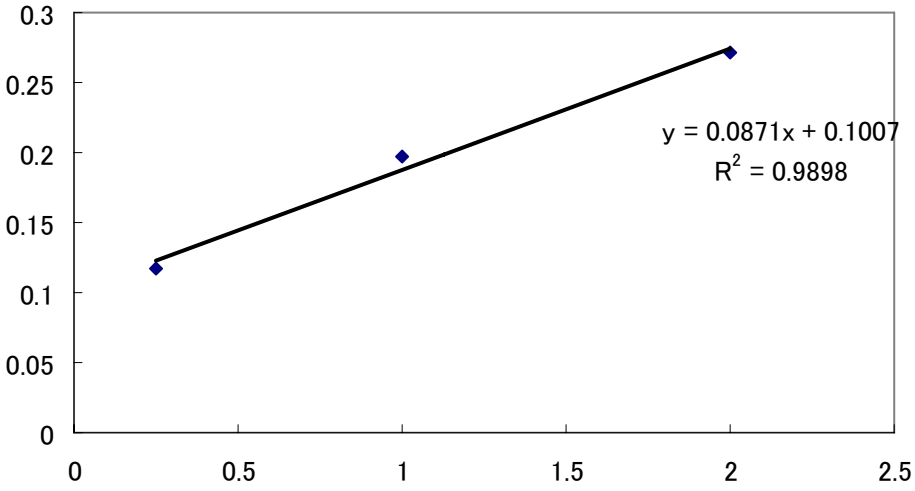

(A)
C6
N2a
boPrP
-
-
+
PrP
(B)
C6
N2a
boPrP
-
-
+
PrP
(C)
boPrP
C6
N2a
-
-
+
PrP
(D)
C6
N2a
boPrP
-
-
+
α-tubulin

Fig. 11

a)



Recombinant bovine PrP (ng)

b)

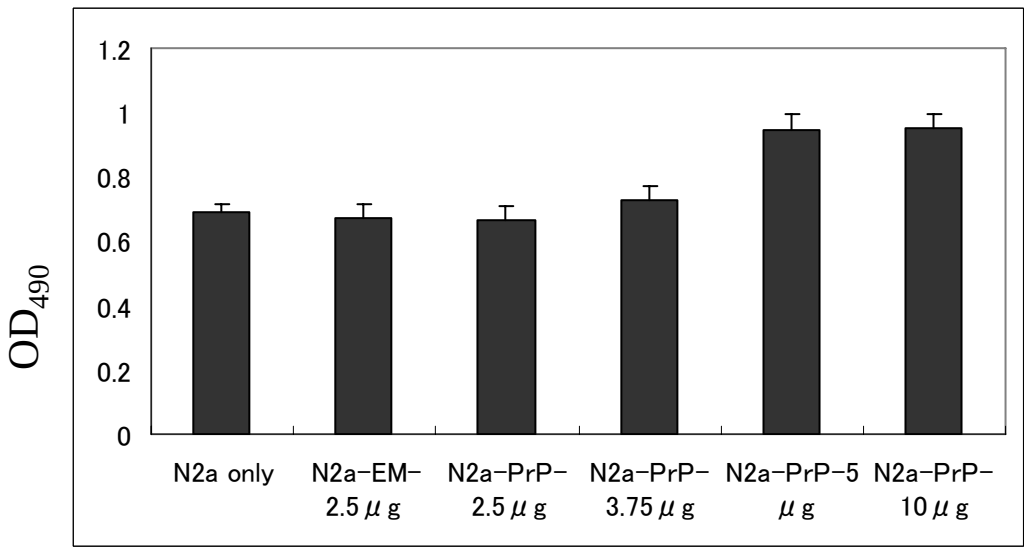


Fig. 12

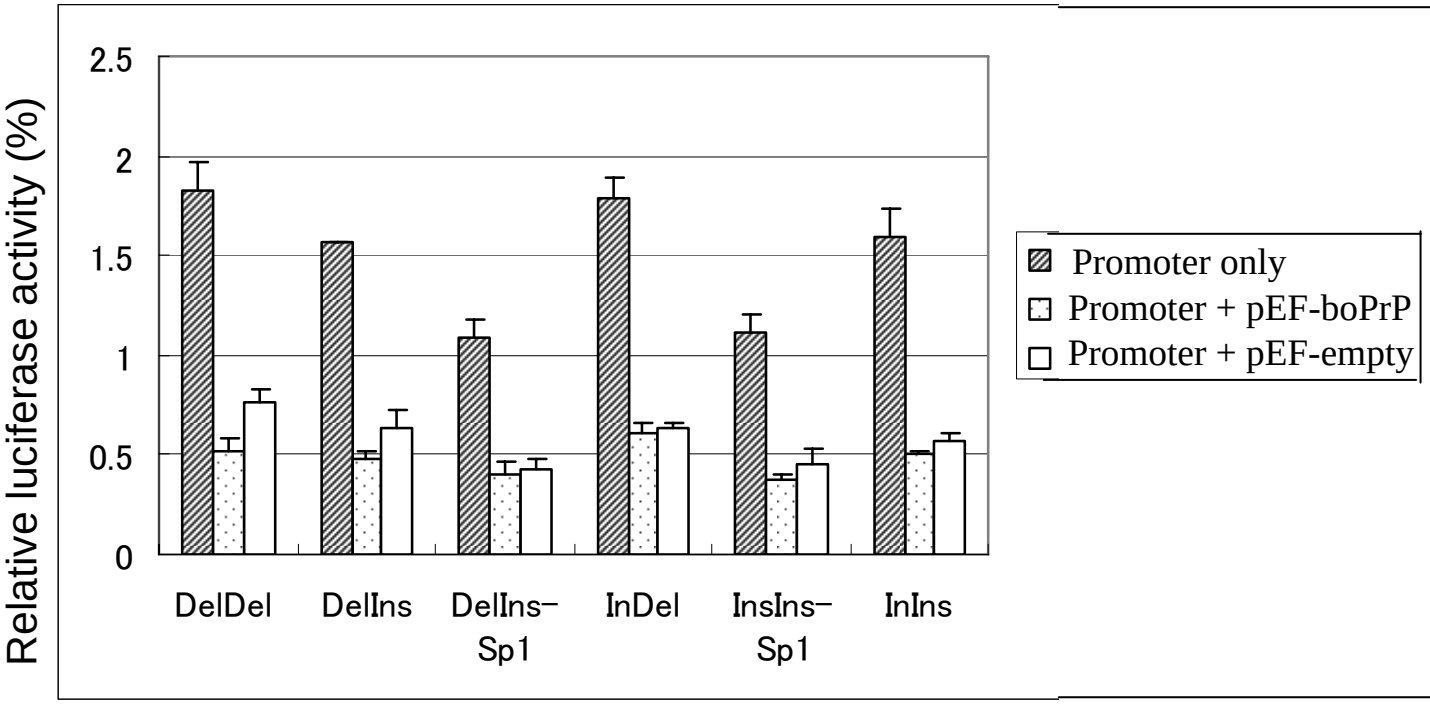

Relative luciferase activity (%)
2.5
2
1.5
1
0.5
0
DelDel
DelIns
DelIns–Sp1
InDel
InsIns–Sp1
InIns
Promoter only
Promoter + pEF-boPrP
Promoter + pEF-empty

Table 2. 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	Bov PRNP-1470R_HindIII	TCAAGCTTTAGCAACTACTCCAAAACTTA	165	47796-47960
BovPRNP-463F_HindIII	AGAAGCTTGAGCAGGAACTGAGTAAATGACGG	BovPRNP+400R_MluI	TAACGCGTCTACCGGTGCGATTCGCCCAT(	864	48967-49830
BovPRNP+2389F_MluI	ATAATTACGCGTTGAGTAGATTCATTAGTGGTT	BovPRNP+2526R_BglII	TCAGATCTGGTTGAAACTGTTCAGTT	138	51819-51956
PRNP49686_Fb	TTACCCTCCTGGTTAGGAG	PRNP49777_Rb	CTAGATTCCTACACACCAC	91/103	49686-49777
PRNP47784_Fb	GTGCCAGCCATGTAAGTG	PRNP47883_Rb	TGGACAGGCACAATGGG	100/123	47784-47883

a Nucleotide position refer to AJ298878
b Primer referenced from Hills et al. [21].

Table 3. Genotype frequency of polymorphisms at putative promoter and intron 1 of the bovine *PRNP* in Japanese Black cattle

Polymorphism	Total	+/+	+/-	-/-
Promoter (23bp indel[a])	44	0.091	0.432	0.477
Intron 1 (12bp indel[b])	44	0.159	0.409	0.432
		C/C	C/A	A/A
Sp1 binding site (SNP[c])	44	0.989	0.011	0

TCTCAGATGTCTTCCCAACAGCA
[a] TCTCAGATGTCCTCCCAACAGCA
[b] GGGGGCCGCGGC
[c] Polymorphism at nucleotide 49383.

Table 4 Allele frequencies.

	Variant			Allele frequency of the variant
	23-bp	12-bp	Sp1[a]	
DelDel	deletion	deletion	No substitution	57
DelIns-Sp1	deletion	insertion	C to A	0
DelIns	deletion	insertion	No substitution	3
InsDel	insertion	deletion	No substitution	0
InsIns-Sp1	insertion	insertion	C to A	1
InsIns	insertion	insertion	No substitution	27

[a] This polymorphism has been documented in our previously study.

論 文 の 内 容 の 要 旨

応用動物科学　専攻
平成 18 年度博士課程　進学
氏　　名　薛　光愛
指導教員名　小野寺　節

論文題目　　Studies on the promoter region of the bovine prion protein gene (*PRNP*)
(ウシプリオン蛋白遺伝子プロモーター領域に関する研究)

プリオン病は、正常プリオン蛋白質 (PrP^{c}) が異常プリオン蛋白質($PrP^{Sc)}$ に構造変換し、異常プリオン蛋白質が脳内に蓄積することにより、神経細胞の機能不全および神経細胞死が引き起こされる致死的な神経変性疾患である。プリオン病には、ヒトにおけるクロイツフェルト･ヤコブ病 (Creutzfeldt-Jakob disease)、ヒツジとヤギにおけるスクレイピー (scrapie)、ウシにおける牛海綿状脳症 (bovine spongiform encephalopathies: BSE) が含まれる。プリオン病の病原体（プリオン）の増殖には正常型プリオン蛋白質の発現が必須である。ヒト、ヒツジではプリオン蛋白遺伝子 (*PRNP*) の蛋白翻訳領域のアミノ酸多型がプリオン病に対する抵抗性と関連があると報告されている。ヒツジにおいては 136 番のアラニンがバリンに置換するとスクレイピーに対する感受性が高くなり、171 番のグルタミンがアルギニンに置換すると抵抗性が増やす。ヒトにおいても、129 番目のアミノ酸多型がvCJD感受性と強い関連性があると報告されている。一方で、ウシにおいて*PRNP*の蛋白翻訳領域のアミノ酸多型は存在するが、プリオン病と関連する多型は存在しないと報告されている。しかしながら、プリオン蛋白質の発現レベルと関係するプロモーター領域とプリオン病抵抗性について、近年プロモーター領域の上流に存在する 23 bp 挿入及び欠損がBSEの感受性と関連があると報告されている。現在まで、*PRNP*の蛋白翻訳領域のアミノ酸多型については研究が盛んに行われていたが、ウシプロモーター領域については十分な研究がなされていない。そこで、本研究では、ウシプリオン蛋白遺伝子プロモーター領域の多型、プロモーター活性、および転写に関わる重要な領域を明らかとすることを目的として以下の研究を行った。

第一章　BSEの原因はスクレイピーに感染させたヒツジ、およびBSEに感染したウシ由来の肉骨粉を経口摂取によるものであると一般的に考えられている。

BSEの原因となるPrP^{Sc}はウシだけではなく、ヒトにも感染性を持っている。そのため、多くの国では食物連鎖からBSE-罹患牛を除去することを目的としており、一つの戦略として挙げられるのはBSE抵抗性ウシの育種である。現在、日本でBSE陽性牛が 35 頭発見されたが、その内黒毛和種が 3 頭である。肉用種が乳用牛に比べ、頭数が多いのにもかかわらず、BSE陽性牛が少ないことから、肉用種にはBSEに対する抵抗性が存在するのではないかと考えられている。そこで本研究では、肉用種の 95%を占める黒毛和種の*PRNP*プロモーター領域の多型を明らかにすることを目的とし、同プロモーター領域の活性について研究を行った。黒毛和種 28 頭脂肪組織材料からDNAを抽出し、目的のプロモーター領域を増幅し、pT7-Blue T ベクターに導入、クローニングをし、塩基配列決定を行った。決定された塩基配列をGenBankのホルスタイン種配列AJ298878 と比較した結果、7 箇所 (-184A→G, -141T→C, -85T→G, -47C→A, -6C→T, +17C→T, +43C→T) で一塩基遺伝子多型 (single nucleotide polymorphism: SNP) が確認された。その中には、これまで報告されているホルスタイン種塩基置換と一致したのが 2 箇所 -184/ 49246(A→G), -85/ 49345(T→G) [AJ298878]含まれていた。そして、新たに黒毛和種において 5 箇所の塩基置換を確認し、そのうち、2 箇所のSp1 結合領域に塩基置換が存在した。これらの塩基置換がプロモーター活性に及ぼす影響を明らかにするために、ルシフェラーゼアッセイを行った。それぞれ 6 つのハプロタイプをルシフェラーゼレポーターベクターに組み込み、マウス神経繊維芽腫細胞N2aに遺伝子導入して 48 時間培養後、プロモーター活性測定を行った。6 つのハプロタイプのうち、Sp1 結合領域で-6C→T塩基置換を示したハプロタイプ及び-141T→C塩基置換を示したハプロタイプはトランスフェクション 48 時間後の発現レベルがワイルドタイプのハプロタイプより有意に低下していた。以上の結果から、黒毛和種*PRNP*プロモーターにはいくつかの多型が存在し、中にはプロモーター活性に影響を及ぼす塩基置換が存在することが明らかとなった。このような塩基置換はBSE抵抗性牛の育種に役に立つと考えられた。

第 2 章　　第 1 章ではウシ *PRNP* 5’側領域に存在する Sp1 結合領域の-6C→T 多型によりウシ *PRNP* プロモーター活性が低下することを明らかにした。さらなる解析の結果、Sp1 結合領域の-47C→A 及び 12bp の挿入/欠失の多型は同調してウシ *PRNP* プロモーター活性に影響を与えることがレポーター遺伝子アッセイにより示された。-47A と 23 bp 欠失/12bp 挿入 あるいは 23bp 挿入 /12bp 挿入を含むハプロタイプは他のハプロタイプ（23bp 欠失/12bp 挿入 or 23bp 挿入/12bp 欠失と-47C）及び野生型のハプロタイプ（23 bp 欠失/12bp 欠失 と-47C）に比べ

て有意に低いプロモーター活性が観察された。さらに、ゲルシフトアッセイの結果、ウシ *PRNP* プロモーター領域の二つの多型が同調して Sp1 が *PRNP* プロモーター領域へ結合する活性に影響を与えることが明らかとなった。これらの結果により、2 箇所の Sp1 結合領域の多型は Sp1 の *PRNP* 領域へ結合及びプロモーター活性をコントロールしているものと考えられた。そして、ウシ *PRNP* プロモーター領域の single nucleotide polymorphisms （SNP）等の変異は、BSE 発病に影響を及ぼす可能性が考えられる。

第 3 章　ウシ *PRNP* プロモーター領域の転写に関わる重要な領域の同定を目的とした。マウス及びウシプリオン蛋白質を発現するベクターをルシフェラーゼレポーターベクターと同時に Lipofectamine 法によって、N2a 細胞に遺伝子導入し、プリオン蛋白質過剰発現する場合のプロモーター領域の活性変化を測定した。その結果、プロモーター活性は導入したプリオン蛋白質量依存的に抑制されることが分かった。そして、プロモーター活性抑制効果は、マウスプリオン蛋白質のほうがウシプリオン蛋白質より、同じ量の発現ベクターを導入した場合強いことが確認された。以上の結果からプリオン蛋白質を過剰発現させた場合、*PRNP* プロモーター領域が抑制されることが明らかとなった。そこで、具体的にどの領域が過剰発現に反応するのかを明らかとするために、いくつかの挿入/欠失を示すプラスミドを用いて同様のプリオン蛋白質過剰発現実験を行った。その結果、23bp 欠失 12 bp 欠失を示した遺伝子断片のプロモーター活性が抑制された。以上のことより、上流の 23bp、下流の 12 bp を含む領域が転写に重要であることが示唆された。

本研究では黒毛和種 *PRNP* プロモーター領域にはいくつかの多型が存在し、中にはプロモーター活性に影響を及ぼす塩基置換が存在することを明らかにした。さらに、ウシプロモーター領域を解析した結果、プロモーター上流の 23bp、下流の 12 bp を含む領域が転写に重要であることが考えられた。このような遺伝的情報は BSE 抵抗性牛の育種に役に立つと考えられた。