Bovine Prion Protein Gene (PRNP) Promoter Polymorphisms Modulate PRNP Expression and May Be Responsible for Differences in Bovine Spongiform Encephalopathy Susceptibility*§

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The susceptibility of humans to the variant Creutzfeldt-Jakob disease is greatly influenced by polymorphisms within the human prion protein gene (PRNP). Similar genetic differences exist in sheep, in which PRNP polymorphisms modify the susceptibility to scrapie. However, the known coding polymorphisms within the bovine PRNP gene have little or no effect on bovine spongiform encephalopathy (BSE) susceptibility in cattle. We have recently found a tentative association between PRPN promoter polymorphisms and BSE susceptibility in German cattle (Sander, P., Hamann, H., Pfeiffer, L., Wemmheuer, W., Brenig, B., Groschup, M., Ziegler, U., Distl, O., and Leeb, T. (2004) Neurogenetics 5, 19–25). A plausible hypothesis explaining this observation could be that the bovine PRPN promoter polymorphisms cause changes in PRPN expression that might be responsible for differences in BSE incubation time and/or BSE susceptibility. To test this hypothesis, we performed a functional promoter analysis of the different bovine PRPN promoter alleles by reporter gene assays in vitro and by measuring PRPN mRNA levels in calves with different PRPN genotypes in vivo. Two variable sites, a 23-bp insertion/deletion (indel) polymorphism containing a RP58-binding site and a 12-bp indel polymorphism containing an SP1-binding site, were investigated. Band shift assays indicated differences in transcription factor binding to the different alleles at the two polymorphisms. Reporter gene assays demonstrated an interaction between the two postulated transcription factors and lower expression levels of the ins/ins allele compared with the del/del allele. The in vivo data revealed substantial individual variation of PRPN expression in different tissues. In intestinal lymph nodes, expression levels differed between the different PRPN genotypes.

Bovine spongiform encephalopathy (BSE) is the bovine analog to variant Creutzfeldt-Jakob disease in humans, scrapie in sheep, chronic wasting disease in elk and deer, feline spongiform encephalopathy in cats, and transmissible mink encephalopathy. The causative agents of these transmissible spongiform encephalopathies are infectious proteins, the so-called prions according to the widely accepted hypothesis of Prusiner (2). Normal host cellular prion protein (PrPc) changes its conformation due to triggering by inoculated scrapie PrPSc. Ingestion of meat and bone meal from scrapie-infected sheep and BSE-infected cattle initiated the large BSE outbreak in the United Kingdom in the last decade of the 20th century (3). Prions are transported eventually from the gastrointestinal tract to the brain, in which spongiform degeneration of brain structure leads to neurodegenerative disorder. There is evidence that the immune system is involved in this process. Specifically, intestinal lymph nodes, B-lymphocytes, follicular dendritic cells, and the spleen are essential for carrying PrPSc to the target organ brain (4–9).

At least for some transmissible spongiform encephalopathies, host genetic factors modulate susceptibility to prion infection. This phenomenon was initially discovered in sheep, in which several mutations within the coding sequence of the prion protein gene (PRNP) are known to lead to increased or decreased scrapie susceptibility (10–14). In humans, a polymorphism at codon 129 of the PRNP coding sequence is strongly correlated with susceptibility to variant Creutzfeldt-Jakob disease, as all human variant Creutzfeldt-Jakob disease patients share the homozygous Met129/Met129 genotype, whereas Val129/Val129 and heterozygous individuals have not been diagnosed with variant Creutzfeldt-Jakob disease so far. However, in cattle, none of the known polymorphisms within the bovine PRNP coding sequence seem to have an influence on BSE susceptibility. Alternatively, it has been 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 (15).

We previously reported the first tentative association of BSE susceptibility with polymorphisms in the PRNP gene promoter (1). In our previous study, the allele frequencies at two linked insertion/deletion (indel) polymorphisms within the bovine PRNP promoter differed significantly between the 48 healthy and 43 BSE-affected German cattle analyzed. The most common haplotypes at these two polymorphisms contained either both insertions (referred to as ins/ins) or both deletions (referred to as del/del). The frequency of the del/del haplotype was higher in the BSE-affected group. We therefore hypothesized that the observed differences in haplotype frequencies may reflect differences in PRNP promoter activity. According to this hypothesis, the del/del allele, which was over-represented in the affected group, should show stronger promoter activity than the ins/ins allele. To test our hypothesis, we investigated the functional properties of different allelic variants of the
bovine PRNP promoter by performing reporter gene assays in vitro and by quantitative real-time (qRT) PCR in vivo.

**EXPERIMENTAL PROCEDURES**

*In Silico Analysis of Transcription Factor-binding Sites*—The computer software MatInspector was used to scan the bovine PRNP promoter region for possible transcription factor-binding sites (16). The region spanned from positions 46754 to 51993 according to the bovine PRNP genomic sequence (GenBank™ accession number AJ298878), including the putative promoter (17). Different allelic variants of the promoter sequence as determined previously (1) were analyzed for differences in transcription factor-binding site content (see Fig. 1A).

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts were prepared from bovine brain and PT cells transfected with the vector pRP58 (see below) following the protocol of Dignam et al. (18). Approximately 50-bp oligonucleotides surrounding polymorphisms −1980T→C, −1594indel23bp, −85G→T, +300indel12bp, +571A→G, and +709A→G were designed with both alleles (see Fig. 1A). The oligonucleotides with the sequence surrounding the polymorphism +571A→G were used for EMSA with the gel shift assay system (Promega, Mannheim, Germany) according to the manufacturer’s instructions. The other oligonucleotides were double-stranded and 32P-labeled and used at 25 fmol for DNA-protein binding reactions with 1 μg of poly(d-I-C), 10 μg of bovine serum albumin, 5× binding buffer (250 mM HEPES/NaOH (pH 7.9), 50 mM MgCl2, 750 mM to 1.5 M NaCl, 5 mM dithiothreitol, 5 mM EDTA, and 25% glycerol), 2.25–8.75 μg of nuclear extract or 300 ng of recombinant human SP1 extract (Promega), and possibly 250 fmol or 2.5 pmol of unlabeled double-stranded oligonucleotide as specific or nonspecific competitor. The binding reactions were incubated for 10 min on ice. Electrophoresis of the samples through a native 8% polyacrylamide gel (19:1 acrylamide/bisacrylamide) in 1× Tris borate/EDTA buffer was followed by autoradiography.

*Promoter-Reporter Gene Constructs*—All constructs prepared are shown in Fig. 1B. A PCR product including 2676 bp of the 5′-flanking sequence, exon 1, intron 1, and the first part of exon 2 of the bovine PRNP gene was cloned as an MluI-BglII fragment into the promoterless reporter vector pGL3-Basic (Promega). The following primers were used for this PCR: 5′-ATA ATT ACG CGT TCA CCA TTT CCG AAT ACA TTC-3′ (forward) and 5′-TAA TTA AGA TCT TGG ATT TGT GTC TCT GGG AAG-3′ (reverse). The template for generating this fragment was the bovine bacterial artificial chromosome clone CH240-12001. The resulting construct represented the new vector pDelDel, with deletions at both −1594indel23bp and +300indel12bp.

The mutant with a 12-bp insertion at +300indel12bp, termed pDelIns, was generated by a splice overlap extension reaction on the template pDelDel (19). The mutant plnDelDel with a 23-bp insertion at −1594indel23bp was made by primer extension ligation on the template pDelDel. The construct with both insertions (plnDelIns) was derived by combining plasmids plnDel and pDelIns using the restriction sites MluI and AatII.

The constructs p12Ins and p12Del were created by elongase PCR amplification (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol by a touch-down protocol (20) using primers 5′-CAA GAG ATC TAG AGA TGC TTC ACT GCC CCC AAC ATG GTG CC-3′ (forward) and 5′-ATT TAG ATC TCT GGG AAC ACA GAT GCT TCG GGG CGG-3′ (reverse). Genomic DNA from an animal homozygous for the 12-bp insertion or DNA from an animal homozygous for the 12-bp deletion was used as a template. The PCR products were cloned into the pGL3-Basic vector.

**In Vivo Analysis of Transcription Factor-binding Sites**—Nucleotide sequences of the primer binding sites were predicted from the bovine PRNP promoter region and synthesized by Invitrogen (Karlsruhe, Germany) using the oligonucleotides with the following sequences: 5′-AAT ATT ACG CGT TCA CCA TTT CCG AAT ACA TTC-3′, 5′-TAA TTA AGA TCT TGG ATT TGT GTC TCT GGG AAG-3′, 5′-ACA TCC-3′, and 5′-ATT TAG ATC TCT GGG AAG ACA GAT GCT TCG GGG CGG-3′. Genomic DNA from an animal homozygous for the 12-bp insertion or DNA from an animal homozygous for the 12-bp deletion was used as a template. The PCR products were cloned into the pGL3-Basic vector.

**Figure 1.** Schematic representation of the 5′-end of the bovine PRNP gene and the reporter gene constructs used in this study. A, the first two untranslated exons of the bovine PRNP gene are indicated as boxes. Six naturally occurring polymorphisms that affect predicted transcription factor-binding sites are indicated above the schematic gene map. The positions of the polymorphisms are given with respect to the transcription start site (position 49430 in GenBank™ accession number AJ298878). Six transcription factors whose binding sites are affected by the polymorphisms are indicated, with the respective alleles containing the binding sites in parentheses. NeuroG1, neurogenin-1. B, six reporter gene constructs in the pGL3-Basic vector were prepared. In these constructs, various alleles of the bovine PRNP promoter drive the expression of firefly luciferase (luc).

The RP58-expressing vector pRP58 was generated by cloning a PCR product with the cDNA from the human RP58 gene into the pGL3-Control vector (Promega), replacing its luciferase box. PCR was carried out on the template clone IRAKp961E13474Q. The clone carries one discrepancy with respect to RP58 (GenBank™ accession number NM_006352), c.324C→T. This potential cloning artifact was corrected by site-directed mutagenesis so that pRP58 conformed to accession number NM_006352. The restriction sites HindIII and XbaI were used for cloning this PCR product into the pGL3-Control vector. The expression vector pRSV/SP1 was a kind gift from M. Wegner (University of Erlangen-Nürnberg). The XbaI fragment from plasmid pSP1-778C (21)
was cloned into the vector pRSV as described (22). All PCRs were carried out with proofreading polymerases, and all constructs were sequenced to verify successful cloning.

**Cell Culture and Transient Transfection Experiments**—The bovine cell lines KOP (esophageal tissue of a calf) and PT (kidney cells of a calf) were obtained from the Friedrich Loeffler Institute (Isle of Riems, Germany). These cell lines were cultured in Dulbecco’s modified Eagle’s medium with stable glutamine and 1 g/liter β-glucuronidase (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (Biochrom AG) at 38 °C and 5% CO₂.

For transient transfection assays, ~6 × 10⁵ PT cells or 3 × 10⁴ KOP cells were seeded 24 h before transfection into 12-well plates (Biochrom AG). Cells reaching 60–80% confluency were transfected using 1.2 μl of Effectene reagent and 1.2 μl of Luciferase reference control plasmid, Promega) were used. If cotransfection with pRSV/SP1 and/or pRP58 (Promega) were performed, 75 ng/well each test construct, 15 ng/well pRL-TK, and 15 ng/well transcription factor-building plasmid, 135 ng/well each test construct and 15 ng/well pRL-TK (Promega) were used. If constructs were transfected without a transcription factor-binding site, 12-bp deletion was used for RNA isolation with the Nucleospin 96 tissue kit (Macherey-Nagel, Duren, Germany). To determine the genotype with respect to the indel polymorphisms —Oligonucleotides surrounding the six selected polymorphism positions were designed and used as DNA Isolation and Genotyping —The bovine PRNP 96 tissue kit (Macherey-Nagel, Duren, Germany). To determine the genotype with respect to the indel polymorphisms —Oligonucleotides surrounding the six selected polymorphism positions were designed and used as molecular probes, the probe sets were arranged over 5 log levels on each plate. The PRNP and GAPDH genes was normalized by dividing it by the bovine GAPDH expression level. All assays were performed in duplicates.

**DNA Isolation and Genotyping**—A second piece of each tissue sample used for DNA iso.

| Polymorphisms within the bovine PRNP promoter that affect transcription factor-binding sites | Transcription factor | Binding site position | Orientation | Consensus binding site | Binding allele | Non-binding allele |
|---|---|---|---|---|---|---|
| POZ (BCL6) | −1980 | + | gcTAGAaa | gcTAGAaa | gcCAGAaa |
| RP58 (ZNF238) | −1594 | − | gaCATCtg | gaCATCtg (23-bp insert) | 23-bp deletion |
| NEUROG1 | −85 | − | CCAAGctg | CCAAGctg | CCAAGccg |
| SP1 | +300 | + | gGGCggg | gGGCggg (12-bp insert) | 12-bp deletion |
| AP2 (TFAP2A) | +571 | − | gcCCCAaggagcgcgc | gcCCCAaggagcgc | gcCCCAaggagcgc |
| EGR4 | +709 | + | GGGTggc | GGGTggc | GGGTggc |

**RESULTS**

**Putative Transcription Factor-binding Sites**—The bovine PRNP promoter region from positions −2676 to +2564 was screened in silico for transcription factor-binding sites. This region comprises 5′-flanking sequence as well as intron 1 because it has been shown that both regions contribute to promoter activity (17).

All alleles for each polymorphic position were screened for transcription factor-binding sites. This led to the identification of 18 differences in putative transcription factor-binding sites between the different alleles. We excluded 12 of these differential sites based on the properties of the potentially binding transcription factors. For example, we did not follow up a differential transcription factor-binding site of hepatic nuclear factor-4, as this transcription factor is involved mainly in liver-specific gene regulation. Consequently, we selected six transcription factor-binding sites that seemed most likely to be functionally relevant for the regulation of PRNP transcription (TABLE ONE).

**Binding of Putative Transcription Factors to Selected Sections of the PRNP Promoter**—Oligonucleotides surrounding the six selected polymorphic transcription factor-binding sites were designed and used as
probes in EMSAs. Every probe was designed with one or the other allele of the polymorphisms described in TABLE ONE.

The most striking differences in transcription factor binding to different PRNP alleles were observed in the case of the 12-bp indel polymorphism at position +300 and the transcription factor SP1 (Fig. 2). The 12-bp insertion allele was able to bind SP1, whereas the 12-bp deletion allele did not bind SP1.

EMSA was also performed with the potential RP58-binding site within the 23-bp indel polymorphisms at position −1594 in the 5′-flanking sequence. Using the same brain nuclear extract as used in the SP1 EMSAs, no band shifts were initially visible (data not shown). However, reproducible band shifts with nuclear extracts from overexpressing cells were obtained (Fig. 3). The experiment showed that the 23-bp insertion allele produced strong and specific band shifts with transcription factor RP58. However, the deletion allele produced only a weak band shift that could be competed with the unlabelled insertion allele, indicating that RP58 has a higher affinity for the insertion allele than for the deletion allele.

The polymorphic transcription factor-binding sites for POZ (position −1980), neurogenin-1 (position −85), AP2 (position +571), and EGR4 (position +709) were also evaluated in similar EMSAs (supplemental Fig. 1). In these four instances, no conclusive differences in the binding properties of the different respective alleles were observed. Both allelic POZ probes produced identical band shift patterns with two shifted bands for each probe. In the case of the −85G→T polymorphism, both allelic probes led to a shifted band of the same size. The shifted band with the G allele had a higher intensity; however, as this allele was predicted to be the non-binding allele for NEUROG1, the polymorphism was not further studied. Both allelic AP2 probes did not produce any band shift, whereas a control oligonucleotide with a perfect AP2 consensus site led to a shifted band of the expected size. In the case of EGR4, both allelic probes produced identical band shift patterns with two bands for each probe. This led to the conclusion that only the 23- and 12-bp indel polymorphisms are involved in differential allelic PRNP promoter modulation and thus in PrP expression. Therefore, our further experiments focused on these two polymorphisms.

Promoter-Reporter Gene Assays—Plasmids pDelDel, pDELIns, plnDel, plnIns, and p12Del carrying the firefly luciferase open reading frame under the control of different PRNP promoter alleles were cotransfected into two different bovine cell lines, PT and KOP, with the pRL-TK normalization vector (Fig. 1). Each assay was carried out in triplicates at 6 (PT) or 5 (KOP) separate weeks (nPT = 18 and nKOP = 15). Every week, cells were also transfected with the pGL3-Control vector for standardization of these six or five passages. The activity of firefly luciferase compared with that of Renilla luciferase reflected the promoter activity. The mean values of each test construct are indicated in Fig. 4.

FIGURE 2. EMSA analysis of SP1 binding to the PRNP promoter sequence. 25 fmol of 32P-radiolabeled double-stranded oligonucleotides (Oligo) corresponding to the two alleles at the 12-bp indel polymorphism in intron 1 were used as probes: +12, 5′-CTC GGA ATG TGG GCG GGG GCC GCG GCT GGC TGG TCC CCC TC-3′ (sense); and −12, 5′-CAT TTA CTC GGA ATG TGG GCT GGC TGG TCC CCC TCC CGA G-3′ (sense). Competitions were performed by the addition of unlabelled double-stranded oligonucleotides (250 fmol or 2.5 pmol) to the binding reaction. The binding buffer contained 0.75 mM NaCl. Purified SP1 protein (300 ng/lane) was used as a positive control. Nuclear extract (NE; 5.25 μg/lane) was prepared from bovine brain. Negative controls (neg) lacked protein. The +12 oligonucleotide probe produced a shift with purified SP1 protein and with nuclear extract from bovine brain. The observed SP1 binding to the +12 oligonucleotide was specific, as it could be competed with the unlabelled +12 oligonucleotide, but not with the nonspecific unlabelled −12 oligonucleotide. The shifted band with nuclear extract was the same size as the shifted band with purified SP1, which also corroborates the identity of the bound protein. The labeled −12 oligonucleotide did not bind SP1.

FIGURE 3. EMSA analysis of RP58 binding to the PRNP promoter sequence. 25 fmol of 32P-radiolabeled double-stranded oligonucleotides (Oligo) corresponding to the two alleles at the 23-bp indel polymorphism in the 5′-flanking sequence were used as probes: +23, 5′-TAG GTA TCA CGT CAT CTC TAC CAA CAG CAG CTT CAG TGG TGG GCC GCG CTC TCG TGA-3′ (sense); and −23, 5′-ATT CCA ACT CCT AGC TAT CAC GTC AAG CCT CAG TGG GCC TGG GCG GGC CTC TGC-3′ (sense). Competitions were performed by the addition of the unlabelled +23 or −23 oligonucleotide (250 fmol or 2.5 pmol) or the unlabelled AP-G oligonucleotide (2.5 pmol; sense, 5′-GCG GTG AGT GCC GGA GCG TGG GCC CCC CAG CCG C-3′) to the binding reaction. The binding buffer contained 1.125 mM NaCl. Nuclear extract (NE; 3.33 μg/lane) was prepared from PT cells transfected with an expression construct for transcription factor RP58. Negative controls (neg) lacked protein. The +23 oligonucleotide produced a strong band shift that could be competed with an excess of the unlabelled +23 oligonucleotide. Competition with the −23 oligonucleotide reduced the intensity of the shifted bands; however, it did not completely abolish binding of RP58 to the +23 oligonucleotide. On the other hand, the −23 oligonucleotide produced a weak band shift with RP58 that could be competed with both the unlabelled −23 and +23 oligonucleotides. The completely unrelated AP-G oligonucleotide was not able to compete with either the +23 or −23 band shift.
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In both cell lines, the p12Ins vector yielded a significantly higher expression level than the p12Del vector \( (p < 0.0001) \). The order of expression levels of the longer reporter gene constructs in PT cells was as follows: pDelDel, pDelIns, pInsDel, pInsIns. In KOP cells, the order was as follows: pDelIns, pDelDel, pInsDel, pInsIns. In both cell lines, the pDelDel vector yielded significantly higher expression levels than the pInsIns vector \( (p_{\text{KOP}} < 0.0001 \) and \( p_{\text{PT}} = 0.0034) \). Thus, the effect of the isolated 12-bp indel polymorphism in the shorter reporter gene constructs p12Ins and p12Del was reversed in the longer reporter gene constructs pInsIns and pDelDel. The pInsDel and pDelIns constructs displayed inconsistent expression levels in the two cell lines. However, it must be kept in mind that pDelIns is a very rare haplotype and that pInsDel has not yet been found in an animal at all, not in the previous studies (1) or in the present qRT-PCR study (see below).

Promoter-Reporter Gene Assays with Overexpression of Specific Transcription Factors—We transfected the PT and KOP cell lines with the pDelDel, pDelIns, pInsDel, pInsIns, p12Ins, and p12Del plasmids, similar to the initial reporter gene assays. In addition to the reporter gene constructs, the transcription factor-expressing plasmids pRP58 and pRSV/SP1 were added to some wells. This study was performed in triplicates at 3 (PT) or 2 (KOP) separate weeks \( (n_{\text{PT}} = 9 \) and \( n_{\text{KOP}} = 6) \) (supplemental Fig. 2).

The addition of pRSV/SP1 to the p12Ins and p12Del vectors resulted in elevated luciferase expression levels with both constructs in PT cells, but only with p12Del in KOP cells \( (p, p_{\text{p12Ins}} = 0.0078 \) and \( p_{\text{p12Del}} < 0.0001; \) and KOP, \( p_{\text{p12Ins}} < 0.0001). In PT cells, the increase in expression was also much more pronounced with p12Del than with p12Ins. The p12Del reporter gene construct includes three putative SP1-binding sites (positions -98 to -93, +351 to +356, and +901 to +906). In addition to these three SP1-binding sites, the p12Ins vector harbors an additional one within the 12-bp insertion at position +300.

In the long constructs, the overexpression of SP1 consistently led to slightly increased luciferase expression if the 23-bp deletion was present at position -1594, regardless of whether the 12-bp indel polymorphism with the SP1 site was present or not. However, in three of four assays with the 23-bp insertion at position -1594, the overexpression of SP1 had no significant effect on luciferase expression. SP1 overexpression also led to an increase in reporter gene expression with pInsIns in the PT cell line. Thus, the presence of the 23-bp insertion in the 5'-flanking sequence seemed to reduce the SP1-induced activation of the PRNP promoter. The overexpression of recombinant Rp58 also increased luciferase expression in all four constructs with the 23-bp deletion at position -1594, whereas the expression level did not change significantly in all four constructs with the 23-bp insertion.

qRT-PCR Experiments—We collected 96 bovine tissue samples from each the brain stem, intestinal lymph nodes, spleen, and liver. RNA was isolated and used for qRT-PCR experiments measuring the absolute transcript quantities of the PRNP and GAPDH genes by the standard curve method. A relative quantification was performed by dividing the PRNP expression level by the GAPDH expression level. DNA from the same tissue samples was used for genotyping with regard to the 23- and 12-bp indel polymorphisms. Five different genotypes were identified: 23del:12del \( (n = 27–30) \), 23del:12het \( (n = 5–6) \), 23het:12het \( (n = 39–41) \), 23het:12ins \( (n = 6–7) \), and 23ins:12ins \( (n = 15–16) \). The results from qRT-PCR were evaluated with respect to these five genotype groups.

The expression levels of the four different tissues differed widely. The highest PRNP mRNA expression levels were encountered in the brain, followed by the spleen, liver, and intestinal lymph nodes. Within these tissue groups, the brain stem, spleen, and liver showed no significant differences in mean expression levels with respect to the genotypes. In lymph node samples, the 23het:12ins expression levels were lower than the 23del:12del, 23del:12het, and 23het:12het expression levels (supplemental Fig. 3). The raw data were not in a normal distribution. Often, single animals showed dramatic variations relative to the average. Intestinal lymph node expression levels of the most common genotypes are shown in Fig. 5. The liver expression levels were an exception; here, the raw data fit better to a normal distribution, and smaller standard errors were observed compared with the other tissues. The complete qRT-PCR data from all tissues are shown in supplemental Fig. 3.

DISCUSSION

In a previous mutational analysis of the bovine PRNP gene (1), we investigated a region that includes the PRNP promoter. We hypothesized that mutations affecting the PRNP expression level might have an influence on BSE incubation time and/or BSE susceptibility. We demonstrated a tentative association of BSE susceptibility with respect to the PRNP genotypes at the 23-bp indel polymorphisms in the 5'-flanking sequence and the 12-bp indel polymorphism in intron 1. The 12-bp indel polymorphism and a couple of single nucleotide polymorphisms within the putative promoter region are in strong linkage disequilibrium with the 23-bp indel polymorphism. We presumed that one of these polymorphisms might have an influence on the promoter activity leading to modified susceptibility.

The mutations -1980T→C, -1594indel23bp, -85G→T, +300indel12bp, +571A→G, and +709A→G were preselected for further experiments with an in silico analysis of transcription factor-binding sites. Allelic variants at these six potential transcription factor-binding sites differ with respect to the consensus binding sites, and the involved transcription fac-
group are indicated by genotypes 23ins:12ins, 23het:12het, and 23del:12del. The mean values of each genotype normalized to bovine GAPDH (determined by the standard curve method. The relative expression levels of PRNP in cattle intestinal lymph nodes with different promoter genotypes. RNA was isolated from 96 bovine tissue samples. Transcript quantities were analyzed by qRT-PCR. Absolute PRNP and GAPDH expression levels were determined by the standard curve method. The relative expression levels of PRNP normalized to bovine GAPDH (PRNP/GAPDH) are shown for all animals with the common genotypes 23ins:12ins, 23het:12het, and 23del:12del. The mean values of each genotype group are indicated by horizontal lines.

FIGURE 5. Expression levels of PRNP in cattle intestinal lymph nodes with different PRNP promoter genotypes. RNA was isolated from 96 bovine tissue samples. Transcript quantities were analyzed by qRT-PCR. Absolute PRNP and GAPDH expression levels were determined by the standard curve method. The relative expression levels of PRNP normalized to bovine GAPDH (PRNP/GAPDH) are shown for all animals with the common genotypes 23ins:12ins, 23het:12het, and 23del:12del. The mean values of each genotype group are indicated by horizontal lines.

tors are themselves expressed in relevant tissues for BSE pathogenesis. These putative transcription factors are SP1, AP2, RP58, NEUROG1, EGR4, and POZ. SP1 is a ubiquitous protein and is involved in the regulation of many promoters (24). AP2 plays a role in tissues of ectodermal origin (25). RP58 was named according to its function and is expressed mainly in the brain (26, 27). It includes a POZ domain, which has been described as an interactor with SP1. It has been suggested that the POZ domain represses transcription by interfering with the DNA-binding activity of SP1 (28). EGR4 (also called nerve growth factor-induced clone C) is like neurogenin-1, a protein that is most likely involved in brain function (29, 30). The transcription factor POZ protein BCL6 plays a role in B-cell differentiation and represses transcription, as does RP58 (31).

All six transcription factors were studied by EMSAs. All oligonucleotide probes with the exception of the two allelic variants of the AP2-binding site produced band shifts. In the case of the two indel polymorphisms, the observed band shifts differed between allelic PRNP promoter variants. The specificity of the DNA-protein interaction was checked by competition with unlabeled probes. In the case of SP1, the identity of the shifted band from the nuclear brain extract was further corroborated by a positive control experiment using purified recombinant SP1. The results of the RP58 EMSA suggest that RP58 had a higher affinity for the 23-bp insertion allele. The weak affinity of RP58 for the 23-bp deletion allele is not surprising, as the RP58-binding site is duplicated with just one mismatch outside the indel polymorphism.

Following the initial evidence from the EMSA experiments, we created reporter gene constructs with these polymorphisms in every possible combination. The 12-bp indel polymorphism in intron 1 was deliberately included in the analysis, as it has previously been shown that intron 1 elements are necessary for bovine PRNP promoter activity (17). Our reporter gene assays revealed an influence of the indel polymorphisms on PRNP transcription rates. Surprisingly, in a short construct, the 12-bp insertion alone resulted in a decreased expression level, whereas in the long construct in combination with the 23-bp insertion, the 12-bp insertion caused a decreased expression level. These findings are compatible with a model in which RP58 binds to the 5′-flanking sequence of the PRNP gene and represses PRNP promoter activity by an interaction with SP1 bound within intron 1, similar to known RP58-SP1 interactions in other promoters (28).

RP58 seems to be a logical candidate for the repressing factor. At this time, the identity of the repressing factor has not been definitely proven. The EMSA results certainly hinted at an involvement of RP58; however, the cotransfection experiments with RP58 were inconclusive. A possible explanation for these results is that the recombinant human RP58 used was indeed able to bind to the recognition sequence within the bovine promoter, but not to exert its repressing function in the heterologous bovine system.

The influence of the PRNP promoter genotype with regard to the 23- and 12-bp indel polymorphisms on PRNP expression in vivo was further studied by qRT-PCR experiments. We chose tissue samples from the spleen, brain stem, and intestinal lymph nodes because of their importance in the infection route of the prions. The liver was chosen as a control organ because it is supposedly not involved in BSE pathogenesis. Only the lymph nodes showed a significant distribution of the five genotype groups. As the expression level of PRNP in intestinal lymph nodes was very low compared with that in the other organs, this result raises the intriguing question of whether the low PRNP amounts in intestinal lymph nodes are rate-limiting for the conversion of cellular PrP<sup>c</sup> into PrP<sup>sc</sup> during the spread of BSE infection toward the central nervous system. The higher expression level of the pDelDel plasmid is consistent with our previous finding that the 23del:12del genotype is associated with higher susceptibility to BSE in German cattle (1).

Our results tentatively indicate that individuals with deletion alleles at the 23-bp indel polymorphism have higher PRNP expression levels in their intestinal lymph nodes. The influence of the promoter polymorphism on the expression levels is rather limited in comparison with the observed variance. This might be because we analyzed whole tissue samples, which may have been composed of varying proportions of different cell types. Future analyses should be performed at the single cell level, e.g. by in situ hybridizations, as it has previously been demonstrated that PRNP expression is not completely uniform within lymph nodes (32). Despite the large variance, the observed differences in PRNP expression levels are consistent with the notion that the 23-bp deletion allele is associated with higher susceptibility to BSE in German cattle.

In conclusion, we have shown that two indel polymorphisms in the bovine PRNP promoter that contain binding sites for the RP58 and SP1 transcription factors modulate the expression level of PRNP in vitro. Our in vivo data show that the PRNP genotype may contribute to the observed high variance of PRNP expression in intestinal lymph nodes.
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