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Cloning and expression of two new prolactin-related proteins, prolactin-related protein-VIII and -IX, in bovine placenta

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Abstract

Background: Prolactin-related proteins (PRPs) are specific proteins of the growth hormone/prolactin (GH/PRL) family in bovine placenta. This study reports the identification and sequencing of a full-length cDNA for two new members of bovine PRPs, bPRP-VIII and -IX, and their localization and quantitative expression in bovine placenta.

Methods: New bPRP-VIII and -IX were identified from bovine placentome. Localization and quantitative gene expression in the placenta were respectively investigated by *in situ* hybridization and real-time RT-PCR methods. Recombinant proteins of these genes were produced by a mammalian HEK293 cell expression system.

Results: Full-length bPRP-VIII and -IX cDNA were respectively cloned with 909 and 910 nucleotide open-reading-frames corresponding to proteins of 236 and 238 amino acids. The predicted bPRP-VIII amino acid sequence shared about 40 to 70% homology with other bPRPs, and bPRP-IX had about 50 to 80% homology of others. The two new bPRPs were detected only in the placenta by RT-PCR. mRNA was primarily expressed in the cotyledon and intercotyledonary tissues throughout gestation. An *in situ* hybridization analysis revealed the presence of bPRP-VIII and -IX mRNA in the trophoblastic binucleate and/or trinucleate cells. bPRP-VIII mRNA was observed in the extra-embryonic membrane on Day 27 of gestation, however, no bPRP-IX mRNA was observed in the extra-embryonic membrane in the same stage of pregnancy by quantitative real-time RT-PCR analysis. Both new bPRP genes were possible to translate a mature protein in a mammalian cell expression system with approximately 28 kDa in bPRP-VIII and 38 kDa in bPRP-IX.

Conclusion: We identified the new members of bovine prolactin-related protein, bPRP-VIII and -IX. Localization and quantitative expression were confirmed in bovine placenta by *in situ* hybridization or real-time PCR. Their different temporal and spatial expressions suggest a different role for these genes in bovine placenta during gestation.

Background

Prolactin-related proteins (PRPs) are members of the growth hormone/prolactin (GH/PRL) family [1]. PRPs are highly expressed in binucleate cells of bovine trophoblasts, although their function is still obscure. No new member had been identified between early 1990 and this year [2]. We recently found a new PRP member through a comprehensive analysis using a bovine utero-placental cDNA microarray [3]. Seven bovine PRP (*bPRP*) genes have been identified in placental tissues, whereas only two of those genes have been shown to be translated. The expression pattern of these genes is spatially and temporally different. *bPRP-I* indicates the considerable importance of regulation of implantation and formation of the placenta in bovines [4,5]. However, *bPRP-VII* was expressed in intercotyledon and cotyledon with rather weak compare to those of *bPRP-I* during the gestation [6].

Placental lactogens (PLs) are known as classical members and PRPs are categorized as the non-classical members of the GH/PRL family in bovine [7]. Various non-classical members are common in rodents, such as mice and rats, and some of them exhibit known biological functions, such as proliferin and prolactin-like protein-A (PLP-A) [7-12]. However, as a whole the biological function of Prolactin-related genes is still not known in various species including bovine. Two research groups identified bovine PRPs mRNA individually; *bPRP-I* to *-III* were identified by the Schuler group [13,14], and *bPRP-IV* to *-VI* were identified by the Nakashima group [15-17]. We recently identified another one named as *bPRP-VII* [6]. Our previous study suggested our bovine placental cDNA library contained other *bPRP* genes [3]. We reveal here the full-length sequences of two new members of *bPRPs*, *bPRP-VIII* and *-IX*, and their localization and quantitative expression. We confirmed the possibility of translation of them and the accuracy of these gene sequences to produce a recombinant protein using the HEK293 cell-transfecting system.

Materials and methods

Animals and tissues

Placental tissues for cDNA cloning and mRNA expression were collected from Japanese Black cows. The extra-embryonic tissues, placenta, and endometrium were collected at a local slaughterhouse on days 27 to 28, 56 to 64, 144 to 149, and 245 to 258 after artificial insemination (day 1). The tissues were separated into four portions: the cotyledon (COT); intercotyledon (the area between the cotyledonary villous (ICOT)); the caruncle area, including the maternal placental septa in the endometrium (CAR); and the intercaruncle area (ICAR). It was difficult to divide the COT and ICOT on days 27 to 28, and thus the COT contained very few villi. Tissues from two different cows on day 27 and one cow on day 28 of gestation (n = 3) were used as Day 27 extra-embryonic membrane

(Day 27EEM), Day 27 caruncle (Day 27CAR), and Day 27 intercaruncle endometrium (Day 27ICAR). Placental tissues were collected on days 56, 58, and 64 (in total, n = 3) and designated as Day 60COT, CAR, ICOT, and ICAR. Sample materials from days 144, 148, and 149 (n = 3) and days 245 (two samples) and 252 (one sample) were marked as Day 150COT, CAR, ICOT, ICAR, Day 250COT, CAR, ICOT, and ICAR. The cotyledonary and caruncular part was separated mechanically, and each part may contained a counter part of tissue. The collected samples were stored at -80 °C until RNA extraction. The placentomes of day 60 were fixed in 3.7% formaldehyde PBS at pH 7.4 and then embedded in paraffin wax and stored at 4 °C until *in situ* hybridization. All procedures for these animal experiments were carried out in accordance with the guidelines and ethics approved by the Animal Ethics Committee of the National Institute of Agrobiological Sciences for the use of animals.

Cloning of full-length *bPRP-VIII* and *-IX* cDNA

The new full-length *bPRP-VIII* and *-IX* cDNA were isolated from bovine cotyledonary tissue by the 3'-rapid amplification of cDNA ends (RACE) method. In brief, a complete RNA was isolated from a bovine placenta on day 60 of gestation using ISOGEN (Nippon Gene, Toyama, Japan). We performed 3'-RACE using a 3'-full RACE core set (Takara, Kyoto, Japan) with *bPRP-VIII*-specific forward primer (5'-CCACAGTCAACAGGAGTCCTCA-3') and *bPRP-IX*-specific forward primer (5'-CCAACAGAGAGTCCTCACCTGCCA-3'). The *bPRP-VIII* and *-IX* primer was designed from bovine EST accession number AW464912 and BP108069, respectively. The 3'-RACE products were sequenced using an ABI Prism 370 automatic sequencer (Applied Biosystems, Foster City, CA, USA) after cloning into a pGEM-T Easy vector (Promega, Madison, WI, USA).

Phylogenetic analysis

Alignments of deduced protein sequences were performed with the multiple alignment software Clustal W 1.83 on the DDBJ web site. Clustal W was also employed to calculate trees using the Neighbor-Joining (NJ) method [18]. TreeView was used to display the phylogenetic tree [19]. The values represent bootstrap scores for 1,000 trials, indicating the credibility of each branch. Except the *bPRP-VIII* and *-IX* sequences, all the *bPRPs* and *bPLs* protein sequences were obtained from GenBank. Their GenBank accession numbers are: *bPRP-I* ([I02944](#)), *bPRP-II* ([M27239](#)), *bPRP-III* ([M27240](#)), *bPRP-IV* ([M33269](#)), *bPRP-V* ([AB239755](#)), *bPRP-VI* ([X59504](#)), *bPRP-VII* ([AB187564](#)), *bPL-Ala* ([I02840](#)), and *bPL-Val* ([M33268](#)).

Three-dimensional structure prediction by FAMS

We predicted the three-dimensional (3D) structure of *bPRP-VIII* and *-IX* by using the FAMS (Fully automated homology modeling system; <http://www.pharm.kitasato->

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1
CCACAGTCAACAGGAGTCCTCACCTGGGATCATTCCACAATCCCC
47
ATG GCT CCA GCT CCC AGC TTC TGT GGA CAC CAG TGG ACT TAC AAC CCT GTC CAA GGG TCC 106
Met Ala Pro Ala Pro Ser Phe Cys Gly His Gln Trp Thr Tyr Asn Pro Val Gln Gly Ser
1
107
TGC CTG CTG CTG CTG CTG GTC ATG TCA AAT CTG CTC CTG TGC CAA GGC AGA ACA TGC CCG 166
Cys Leu Leu Leu Leu Leu Val Met Ser Asn Leu Leu Leu Cys Gln Gly Arg Thr Cys Pro
21
167
TTC TGC TGT TCT GAC GTG TTT GAC ATC CCC TTG GAA TCC CTT AGA AAC CTG TTT CTC AAT 226
Phe Cys Cys Ser Asp Val Phe Asp Ile Pro Leu Glu Ser Leu Arg Asn Leu Phe Leu Asn
41
227
GCT ACC ATG TTG TCC CGC GAC ATT GCC ATC CAT TCC AGC ATA ATG TTC ACT GAT TTT GAT 286
Ala Thr Met Leu Ser Arg Asp Ile Ala Ile His Ser Ser Ile Met Phe Thr Asp Phe Asp
61
287
GAA AAA TAT GCC CAG GGC AAA CAG TAC GAT ATC AAT GAC AAG AGC TGC CAT ACC GAT CCC 346
Glu Lys Tyr Ala Gln Gly Lys Gln Tyr Asp Ile Asn Asp Lys Ser Cys His Thr Asp Pro
81
347
CTC CCT ACT CCC GAA GAA AGA GAA CAA GCC CTA AAG ATA AAC AAT GAA GAC CTG AGT AAG 406
Leu Pro Thr Pro Glu Glu Arg Glu Gln Ala Leu Lys Ile Asn Asn Glu Asp Leu Ser Lys
101
407
TGG ATA ATC ATG TTA CTG TAC TCC TGG AAA AGA CCT CTA AAT CAT CTA GTC ACA GAG CTG 466
Trp Ile Ile Met Leu Leu Tyr Ser Trp Lys Arg Pro Leu Asn His Leu Val Thr Glu Leu
121
467
CAG AAT ATG AAA GAA GTC TCA GAT ACT ATC CTA TCA AGT GCC AGA CAA AAT TTG AAA AAA 526
Gln Asn Met Lys Glu Val Ser Asp Thr Ile Leu Ser Ser Ala Arg Gln Asn Leu Lys Lys
141
527
GTA CAA GAA CTT CAA GAA TTC ATA GAG AGG CAA TTC AGC CAG ATT ATT TTT CCA GTA AGG 586
Val Gln Glu Leu Gln Glu Phe Ile Glu Arg Gln Phe Ser Gln Ile Ile Phe Pro Val Arg
161
587
CGG CTG ACC GAG GGT GAA ATA GTC TGG TCA GAA CTT CCA TCC CTG GTG TCC AGC GAT GAA 646
Arg Leu Thr Glu Gly Glu Ile Val Trp Ser Glu Leu Pro Ser Leu Val Ser Ser Asp Glu
181
647
GAT ATT CGT CAT TCT GCA TTT TAT AAC CTG TTC AAG TGC CTG CAG AGG GAT TCA CAT AAA 706
Asp Ile Arg His Ser Ala Phe Tyr Asn Leu Phe Lys Cys Leu Gln Arg Asp Ser His Lys
201
707
GTT GAC ATG TAC ACC AAG ATC ATG TTG TGC CGA ATC AAC AAC ACG TGC TAA 757
Val Asp Met Tyr Thr Lys Ile Met Leu Cys Arg Ile Asn Asn Thr Cys ***
221
758
ATCCAAAGCATCCCATCCAGCTCTGAGGTGGTGGTAATGTTCTATCCCATAGCAAGCTTCTTAGAAATTTATAGTTTT 836
837
TAATGCATGCTCTGTGAAATGGGTCTCCTCTAAAAAAATAAACACAGACTCTGTAGAAAAAAAAAAAAAAAAA 906

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Figure 1
Nucleotide and deduced amino acid sequences of bPRP-VIII. The arrow indicates the putative primary cleavage site of the signal peptide. The potential N-glycosylation site is underlined with a dotted line. The asterisks indicate the termination codon. The polyadenylation signal is underlined with a solid line.

Table 1: Oligonucleotide primers used for RT-PCR

Gene	Primer	Sequence	Position
<i>bPRP-VIII</i> (AB196438)	Forward	5'-AGAACATGCCCGTTCTGCTGT-3'	155-175
	Reverse	5'-TTAGCACGTGTTGTTGATTCG-3'	757-737
<i>bPRP-IX</i> (AB204881)	Forward	5'-AACTCATGCCCATCCTGTGGT-3'	150-170
	Reverse	5'-TTAGCACTTTTTCGGGATTCG-3'	758-738
<i>GAPDH</i> (U85042)	Forward	5'-CCTTCATTGACCTTCACTACATGGTCTA-3'	71-98
	Reverse	5'-GCTGTAGCCAAATTCATTGTCGTACCA-3'	927-901

u.ac.jp/fams/index.html) [20]. FAMS is the software which predicts 3D model of the target protein from the structural known protein of high homology. In case of *bPRP-VIII* and *-IX*, the 3D structure was constructed based on the human prolactin (hPRL) 3D structure (Protein Data Bank ID: 1N9D) in the element. FAMS program only requires an amino acids sequence as input, and constructs 3D model structures automatically. Visualization of the 3D structure was performed using the RasMol 2.7.3 software <http://www.bernstein-plus-sons.com/software/ras/mol/> [21].

RT-PCR

Tissue distribution of *bPRP-VIII* and *-IX* expression was studied by RT-PCR. Bovine GAPDH was used as a positive control for the PCR. Details of the RT-PCR method were described in previous reports [6,22]. The total RNA in a total reaction mixture was used for reverse transcription and template cDNA synthesis using oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at 42°C for 50 min. Each PCR contained the cDNA template, primers, deoxynucleotide triphosphate mixture (dNTP), MgCl₂, 10 × PCR buffer II, autoclaved milliQ water, and AmpliTaq gold DNA polymerase (Applied Biosystems). Amplification conditions included denaturation at 95°C for 30 sec and extension at 72°C for 1 min. Twenty-seven cycles were performed for all samples. The annealing temperature was set at 60°C for 30 sec. A single denaturation step at 95°C for 10 min before the first PCR cycle and a final extension step at 72°C for 10 min after the last PCR cycle were also performed. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The primers encoding for the *bPRP-VIII* and *-IX* sequences were designed using the sequence illustrated in Fig. 1. The primer sequence was selected to include a mismatch for other bPRPs. The designated primers are listed in Table 1. All the primers were commercially synthesized (Especc Oligo Service, Tsukuba, Japan).

In situ hybridization

Full-length cDNA of *bPRP-VIII* and *-IX* was used as a template for hybridization probe synthesis. Digoxigenin (DIG)-labeled antisense and sense-complementary RNA

probes were prepared as described in previous studies [6,23]. The placentomes were sectioned into 7 μm-thick sections for hybridization. *In situ* hybridization was performed using automated Ventana HX System Discovery with a RiboMapKit and BlueMapKit (Ventana, Tucson, AZ, USA) [6]. Briefly, sections were hybridized with DIG-labeled probes in RiboHybe (Ventana) hybridization solution at 65°C for 6 hours. The sections were washed three times in RiboWash (Ventana) (65°C, 6 min) after hybridization and were fixed in RiboFix (Ventana) (37°C, 10 min). The hybridization signals were then detected with monoclonal-anti-digoxin biotin conjugate (Sigma, Saint Louis, MI, USA). The hybridized glasses were observed after preparation with a Nikon ECLIPSE E800 photomicroscope (Nikon, Tokyo, Japan).

Real-time RT-PCR

Gene expression of *bPRP-VIII* and *-IX* was confirmed quantitatively at each stage of gestation (Days 27FM, 27CAR, 27ICAR, 60COT, 60CAR, 60ICOT, 60ICAR, 150COT, 150CAR, 150ICOT, 150ICAR, 250COT, 250CAR, 250ICOT, and 250ICAR) by real-time RT-PCR analysis. Details of the real-time RT-PCR procedures have been described in previous reports [6,24]. Briefly, fifty ng of the total RNA were reverse transcribed into cDNA for 30 min at 48°C by MultiScribe™ reverse transcriptase with an Oligo dT primer, dNTP mixture, MgCl₂ and RNase inhibitor. Primer pairs and oligonucleotide probes labeled with a reporter fluorescent dye at the 5'-end and a quencher fluorescent dye at the 3'-end were designed using the Primer Express computer software program (Applied Biosystems). The primers and probes for each gene are listed in Table 2. The thermal-cycling conditions included initial sample incubation at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. The cycle threshold values (C_T) indicate the quantity of the target gene in each sample and were determined in real time using an ABI Prism 7700 sequence detector (Applied Biosystems). The relative difference in the initial amount of each mRNA species (or cDNA) was determined by comparing the C_T values. The standard curves for each gene were generated by serial dilution of plasmid containing *bPRP-VIII*, *-IX*, or *GAPDH* cDNA to quantify the mRNA concentrations. The ratio of *bPRP-VIII*

Table 2: Oligonucleotide primers and TaqMan probes used for real-time RT-PCR analysis

Gene	Primer or TaqMan probe	Sequence	Position
<i>bPRP-VIII</i> (AB196438)	Forward	5'-CAAGGGTCTGCCTGCTG-3'	98-115
	Reverse	5'-GGCATGTTCTGCCTTGGC-3'	164-344
	Probe	5'-TGCTGCTGGTCATGTCAAATCTGCTCC-3'	117-143
<i>bPRP-IX</i> (AB204881)	Forward	5'-ATATGCCAGGGCAAAGTGT-3'	287-306
	Reverse	5'-TCGGGAGCATGGAAGGAAT-3'	358-340
	Probe	5'-TATCAATGCCACCAACAGCTGCCACA-3'	311-336
<i>GAPDH</i> (U85042)	Forward	5'-AAGGCCATCACCATCTTCCA-3'	178-197
	Reverse	5'-CCACTACATACTCAGCACCAGCAT-3'	253-230
	Probe	5'-AGCGAGATCCTGCCAACATCAAGTGG-3'	200-225

TaqMan probes of *bPRP-VIII* and *-IX* have 5'-FAM dye and 3'-TAMRA dye
TaqMan probe of *GAPDH* has 5'-VIC dye and 3'-TAMRA dye

and -IX mRNA to *GAPDH* mRNA was calculated to adjust for any variations in the RT-PCR reaction. All values are presented as mean \pm SD. Statistical analysis was performed using one-way ANOVA followed by the Tukey-Kramer multiple comparison test. Differences were considered significant at $P < 0.05$.

Production of recombinant proteins

The *bPRP-I*, *-VIII*, *-IX*, and *bPL* sequences encoding the mature protein region, which combined the FLAG and 6 \times His epitope tag sequences, were inserted into the pFLAG-CMV-3 vector (Sigma). The constructed plasmid was transfected into HEK 293 cells using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) for transient transfection. Stably transfected HEK 293 cells were adapted to the suspension culture in a spinner flask using 293 SFM II medium (Invitrogen, Gibco) and cultured in an atmosphere of 5% CO₂ in air at 37°C for 3 days. The medium was separated by centrifugation and stored at -30°C.

Western blot analysis

The 10 μ g of proteins from the HEK293 cell conditioned media were loaded on each lane, separated by SDS-PAGE, and electrophoretically transferred onto a polyvinylidene difluoride membrane [25]. Western blotting was performed by the method of Towbin et al. [26]. Briefly, the membrane was blocked in 10% skim milk overnight, incubated with anti-FLAG M2 (Sigma) for 1 h at room temperature, followed by incubation with anti-mouse IgG conjugated with alkaline phosphatase (Sigma) (diluted 1:3000) for 1 h at room temperature. Immunopositive bands were stained using NBT (Bio-Rad, Hercules, CA, USA) and BCIP (Bio-Rad).

Results

Sequences of *bPRP-VIII* and *-IX* cDNA

Full-length *bPRP-VIII* and *-IX* were cloned from bovine placenta. The 906- and 910-nucleotide sequences were isolated in *bPRP-VIII* and *-IX*, respectively (Fig. 1 and 2). The protein sequence regions (CDSs) were composed of

711 nucleotides in *bPRP-VIII* and 717 nucleotides in *bPRP-IX*. The 3'-untranslated region contains one AATAAA polyadenylation signal start 20 and 26 bases upstream from the poly (A) addition site in *bPRP-VIII* and *-IX*, respectively. The amino acid sequences deduced from full-length *bPRP-VIII* and *bPRP-IX* cDNA are amino acids 236 and 238. The homology of predicted amino acid sequences of *bPRP-VIII* and *-IX* protein were shown in Fig. 3. The predicted sequence of *bPRP-VIII* protein was 69% homologous to that of *bPRP-VI*, 66% homologous to that of *bPRP-VII*, 61% homologous to that of *bPRP-I* and *-III*, 58% homologous to that of *bPRP-IV* and *-V*, 57% homologous to that of *bPRP-IX*, 42% homologous to that of *bPRP-II*, and 39% homologous to that of *bPL-Ala* (Fig. 3). The predicted sequence of *bPRP-IX* protein was 81% homologous to that of *bPRP-IV*, 76% homologous to that of *bPRP-I*, 70% homologous to that of *bPRP-II*, 60% homologous to that of *bPRP-VII*, 57% homologous to that of *bPRP-VI* and *-VIII*, 53% homologous to that of *bPRP-III* and *-V*, and 40% homologous to that of *bPL-Ala* (Fig. 3). In the phylogenetic analysis, it was shown that *bPRP-VIII* was close to *bPRP-III*, *bPRP-VI*, and *bPRP-VII* sides in the phylogenetic tree and *bPRP-IX* was close to *bPRP-II* and *bPRP-IV* sides in the phylogenetic tree (Fig. 4). The *N*-terminal regions of the *bPRP-VIII* and *-IX* proteins were rich in hydrophobic amino acid residue, which is characteristic of the signal peptide. *bPRP-VIII* had two consensus sequences for *N*-glycosylation and Asn-X-Ser/Thr at the positions of 60 to 62 and 233 to 235 (Fig. 1). *bPRP-IX* also had four consensus sequences for *N*-glycosylation at the positions of 70 to 72, 92 to 94, 146 to 148, and 160 to 162 (Fig. 2). Another atypical *N*-glycosylation site, Asn-X-Cys was found in only *bPRP-IX* at the position of 95 to 97, and this region is identified in *bPLs*. The TAA stop codon was used in both *bPRP-VIII* and *-IX*, and appeared after the sequence TGC, which was present in other *bPRPs* except for *bPRP-VI* and *bPLs* that encode C-terminal cysteine residue [17]. The predicted 3D structures of *bPRP-VIII* and *-IX* mature region are shown in Fig. 5. The structural differences of *N*-glycosylation site,

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1
CCAACAGAGAGTCTCACCTGCGATCATTCTCAATCCCC
41

42
ATG GCT CCA GCT CCC AAC TTC CAT GGA CAC CAG TGG ACT TAC AAC CCT GTC CGA GGG TCC
Met Ala Pro Ala Pro Asn Phe His Gly His Gln Trp Thr Tyr Asn Pro Val Arg Gly Ser
1 20
102
TGC CTG CTG CTG CTG CTG GTC GTG TCT AAT CTG CTC CTG TGC CAA GGC AAC TCA TGC CCA
Cys Leu Leu Leu Leu Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Asn Ser Cys Pro
21 40
162
TCC TGT GGT CCT AAC GTG TTT GTC TCC TTA CGG AAA TCC CTT ACA GAC TTG TAT ATT GAT
Ser Cys Gly Pro Asn Val Phe Val Ser Leu Arg Lys Ser Leu Thr Asp Leu Tyr Ile Asp
41 60
222
GCC GCC TGG CTC TCC CAT GAA TTC CAT AAC CTT TCC GCA ATA ATG TTC AGT GAG TTT AAA
Ala Ala Trp Leu Ser His Glu Phe His Asn Leu Ser Ala Ile Met Phe Ser Glu Phe Lys
61 80
282
GAA AAA TAT GCC CAG GGC AAA CTG TAC CAT ATC AAT GCC ACC AAC AGC TGC CAC ACC AAT
Glu Lys Tyr Ala Gln Gly Lys Leu Tyr His Ile Asn Ala Thr Asn Ser Cys His Thr Asn
81 100
342
TCC TTC CAT GCT CCC GAA GAA AGA GAT AAA GCC CAC CAG ATG AAT AAT GAA GAC CTT AGT
Ser Phe His Ala Pro Glu Glu Arg Asp Lys Ala His Gln Met Asn Asn Glu Asp Leu Ser
101 120
402
AAG TGG ACA CTT GTG TTA CTG TAC TCC TGG AAT AAT CCT CTG TAT CAT CTA GTC CCG AGG
Lys Trp Thr Leu Val Leu Leu Tyr Ser Trp Asn Asn Pro Leu Tyr His Leu Val Pro Arg
121 140
462
CTT CGG AAT ATG AAA AAT CTG TCA GAG GCT GTC GTA TCA AGT GCC ATG GAG ATT GAG AAC
Leu Arg Asn Met Lys Asn Leu Ser Glu Ala Val Val Ser Ser Ala Met Glu Ile Glu Asn
141 160
522
ATG TCA GAC AAA CTT CAG GCA TTC ATA GAG AGT CAA TTC AGG AAG GTT ATT GTT TCA GTC
Met Ser Asp Lys Leu Gln Ala Phe Ile Glu Ser Gln Phe Arg Lys Val Ile Val Ser Val
161 180
582
TTG AAG ACG ATA CAT GAG GCT CGC AGT AGC TGG TCA GGA CTC CCA TCC CTG ACG TCC AGC
Leu Lys Thr Ile His Glu Ala Arg Ser Ser Trp Ser Gly Leu Pro Ser Leu Thr Ser Ser
181 200
642
GAT GAA GAT AGG CGT CTT TCT GAA TTT TAT AAC CTG TTC CAC TGC CTG CAC AGG GAT TCA
Asp Glu Asp Arg Arg Leu Ser Glu Phe Tyr Asn Leu Phe His Cys Leu His Arg Asp Ser
201 220
702
GGG AAA GTT GAC ACG TAC ATC AAG ATC CTG ACG TGC CGA ATC CGC AAA AAG TGC TAA
Gly Lys Val Asp Thr Tyr Ile Lys Ile Leu Thr Cys Arg Ile Arg Lys Lys Cys ***
221 238
759
ATCCACATCCATCCCATCCAGCTCTGAGATGGTCATAATCATCTATCCCATAGCGGGCTACTTGAAATTACAGCTTTTT
837

838
AATGCATGCTCTGTAATGGGCTTCTCTTAAAAATAAACACAGATGGTTTAGAAATGCAAAAAAAAAAAAAA
910

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Figure 2
Nucleotide and deduced amino acid sequences of *bPRP-IX*. The arrow indicates the putative primary cleavage site of the signal peptide. The potential *N*-glycosylation site is underlined with a dotted line. The asterisks indicate the termination codon. The polyadenylation signal is underlined with a solid line.

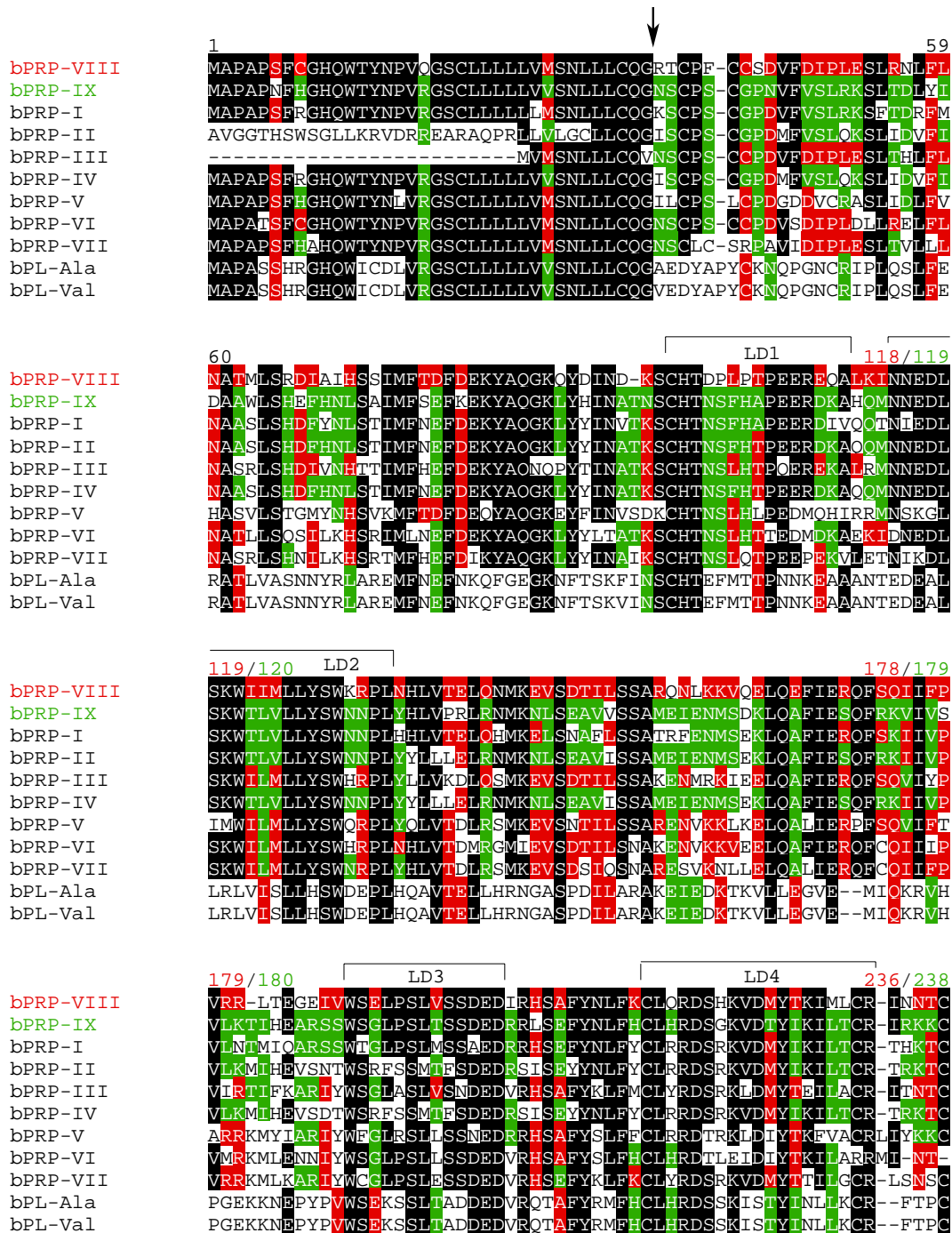


Figure 3
Comparison of amino acid sequences of bPRP-VIII and -IX with other members of bPRPs and bPLs. Residue present in both bPRP-VIII and -IX is shown in black boxes. Residue present in only bPRP-VIII is designated in red boxes. Residue present in only bPRP-IX is designated in green boxes. Amino acid sequences were aligned with assistance from Clustal W 1.83 on the DDBJ web site. The arrow indicates the putative primary cleavage site of the signal peptide of bPRP-VII. LD1, 2, 3, and 4 refer to the conserved domain in the prolactin family.

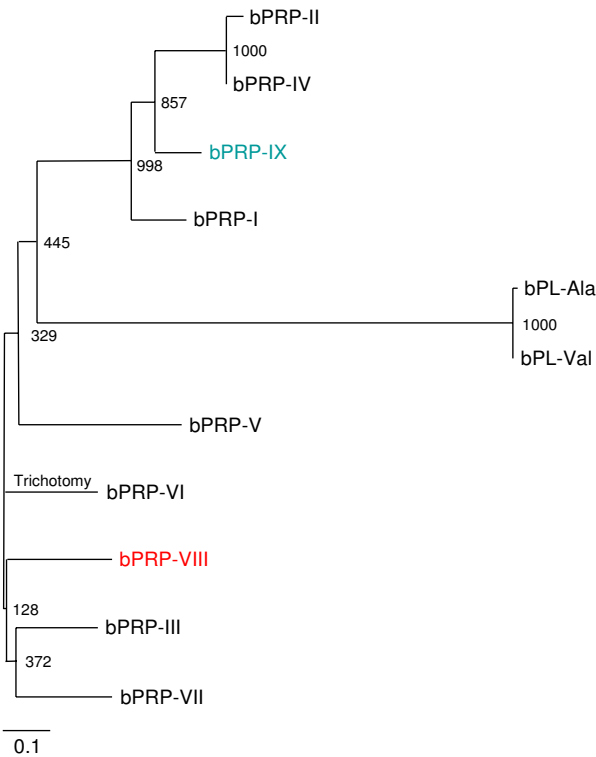


Figure 4
Phylogenetic tree of bPRPs and bPLs. The tree was constructed using TreeView following alignment of the protein sequences by the Clustal W 1.83 algorithm. The numbers at the base of each branch division represent bootstrap values after 1000 repeats. Scale bar represents 0.1 amino acid replacements per amino acid site. For GenBank/DDBJ accession numbers, refer to Materials and Methods.

disulfide bond (-S-S-) and each atomic configuration were confirmed. We submitted these sequences to the DNA Data Bank of Japan (DDBJ). The DDBJ/GenBank accession Nos. are [AB196438](#) and [AB204881](#).

Expression of bPRP-VIII and -IX mRNA in bovine placenta

The distribution of bPRP-VIII and -IX mRNA was examined in various bovine tissues by RT-PCR. Both bPRP mRNAs appeared only in the placental tissue (Fig. 6).

bPRP-VIII and *-IX* mRNA localization was determined by *in situ* hybridization in the bovine placentome on day 60 of gestation (Fig. 7). DIG-labeled *bPRP-VIII* and *-IX* anti-sense RNA probes specifically detected the mRNA transcript in the placentome and intercotyledonary membrane. Both *bPRPs* appeared in the binucleate cells in the cotyledon and intercotyledonary membrane, and *bPRP-IX* mRNA was also detected in trinucleate cells (Figs. 7D and 7F). No significant signals were detected with sense probes in any genes (Fig. 7B and 7E).

Quantitative real-time RT-PCR analysis indicated that *bPRP-VIII* mRNA appeared primarily in the extra-embryonic membrane of Day 27, cotyledon and intercotyledonary membrane of Day 60 to 250 (Fig. 8). The expression intensity increased approximately four-fold in the cotyledon from Day 27 to Day 60, and then slightly decreased until Day 250 (approximately 0.6-fold). The expression intensity increased approximately three-fold in the intercotyledon from Day 27 to Day 60 and was maintained from Day 60 to Day 150. Then it then decreased slightly until Day 250 (approximately 0.7-fold). In the caruncular area, the expression increased by about four to five times by Day 60, and the expression level was maintained to late gestation. However no significant expression was detected in the intercaruncle. *bPRP-IX* mRNA was also detected in the cotyledonary placentome and intercotyledonary membrane after Day 60 of gestation, but it did not appear in the extra-embryonic membrane on Day 27 of gestation. The mRNA expression intensity increased approximately 1.2-fold from Day 60 to Day 150 in the cotyledon and approximately 1.9-fold the intercotyledon, and then those values were maintained until Day 250. No expression of *bPRP-IX* was observed in the caruncular area on Day 27, but stable expression intensities were detected from Day 60 to Day 250. These expression intensities were rather strong compared to the intercotyledonary and cotyledonary areas, even though the sources for this gene may be binucleate and trinucleate cells.

The recombinant proteins of bPRP-VIII and -IX raised with HEK293 cell were detected by Western blot analysis (Fig. 9). The intense bands of bPRP-VIII with a FLAG epitope tag migrated to approximately 28 kDa molecular weight, while those of bPRP-IX with a FLAG epitope tag migrated to 38 kDa, 34 kDa and 26 kDa (Fig. 9).

Discussion

In the present study, we identified two new members of *bPRP* cDNAs, *bPRP-VIII* and *-IX*. Totally nine *bPRP* genes have been identified at this moment in bovine.

bPRP-VIII and *-IX* cDNA sequences had following features, respectively. The position of the polyadenylation signal (AATAAA) in the *bPRP-VIII* started 20 bases upstream from the poly(A) additional site. This configuration is specific to *bPRP-VIII*. The number of bases in *bPRP-VIII* from the polyadenylation signal to poly(A) site are the fewest in known *bPRPs*. The position of the polyadenylation signal in the *bPRP-IX* started 26 bases upstream from the poly(A) additional site. This position coincides with those in *bPRP-II* and *bPRP-IV* [14,15]. The signal peptide sequences in the N-terminal regions of both bPRP-VIII and -IX were conserved similarly to those in other bPRPs/bPLs (Fig. 3). The signal cleavage site was predicted to be between the Glu-36 and Arg-37 in bPRP-VIII, and

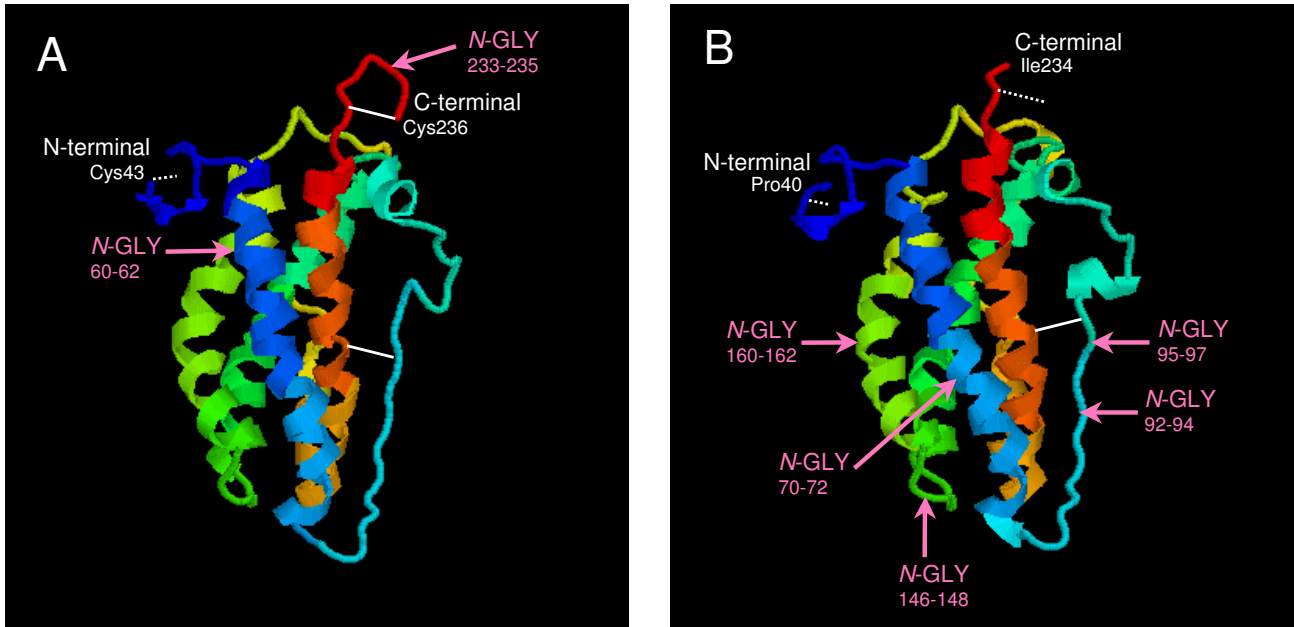


Figure 5
The predicted 3D structure of (A) bPRP-VIII and (B) bPRP-IX mature protein. The 3D structure were predicted by FAMS software. bPRP-VIII structure was able to construct from Cys43 to Cys236 amino acid region. bPRP-IX structure was able to construct from Pro40 to Ile234 amino acid region. Disulfide bonds refer to white solid line. Predicted disulfide bonds refer to white dot line. N-GLY refer to potential N-glycosylation site.

between Glu-36 and Asn-37 in bPRP-IX by homology to the N-terminal regions of the bPRPs/bPLs (Fig. 3). As a result, bPRP-VIII is predicted to be a mature protein composed of 200 amino acids, while bPRP-IX has 202 amino acids. bPRP-VIII lacked two amino acids (positions 94 and 183), which is a sequence characteristic, since other known bPRPs have 202 amino acids. bPRP-VIII had two potential N-glycosylation sites (amino acid portions 60 to 62 and 233 to 235, Figs. 1, 3 and 5). The position of 60 to 62 coincided with those of bPRP-III, -VI, and -VII [6,14,17], however, the second N-glycosylation site was found at 233 to 235 in amino acids. In contrast, bPRP-IX had four potential typical N-glycosylation sites of Asn-X-Ser/Thr (amino acid portions 70 to 72, 92 to 94, 146 to 148, and 160 to 162, Figs. 2, 3 and 5). All these configurations of Asn-X-Ser/Thr coincide with those of bPRP-II and -IV [14,15]. However, it is characteristic that only the bPRP-IX have atypical N-glycosylation site of Asn-X-Cys (amino acid portions 95 to 97). The conserved domains (LD1 to LD4, Fig. 3) were suggested by Yamakawa et al. [15] in other prolactin-related genes. The bPRP-VIII and -IX sequences also revealed these LD domains. Cysteine residues (PRP-VIII positions 96, 213, and 230, and PRP-IX positions 97, 215, and 232) in the LD1 and LD4 domains were conserved in bPRPs/bPLs, except for bPRP-VI. bPRP-

VIII had four more cysteines (positions 38, 41, 42, and 236, Fig. 3, a total of seven residues in mature protein), and bPRP-IX had three more cysteines (positions 38, 41, and 238, Fig. 3, a total of six residues in mature protein).

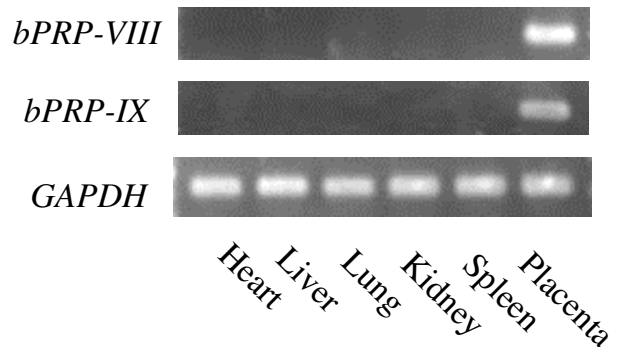


Figure 6
Expression of bPRP-VIII and -IX mRNA in bovine tissues. Heart, liver, lung, kidney, and spleen were used by RT-PCR. Cotyledonary tissue at day 148 of gestation was used as a placental sample. GAPDH expression in each tissue is presented as standard data.

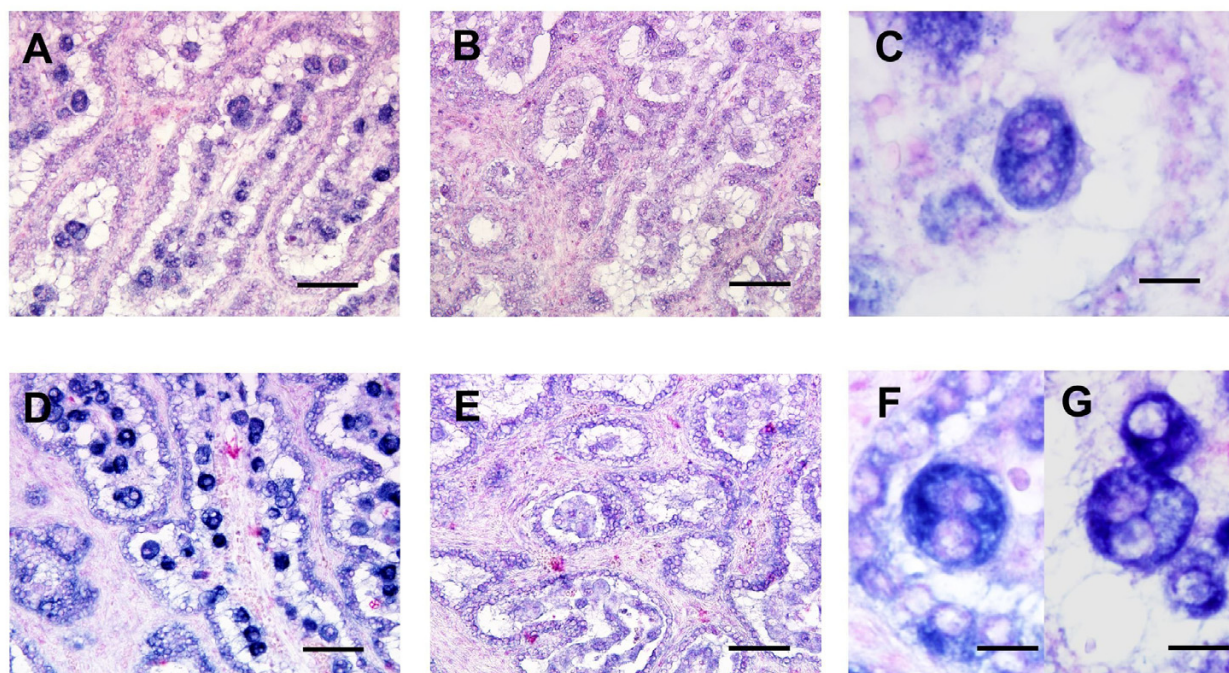


Figure 7

Localization of *bPRP-VIII* and *-IX* in the bovine placentome on day 60 of gestation. (A, B, C) *bPRP-VIII* and (D, E, F, G) *bPRP-IX* mRNA were detected by *in situ* hybridization. (A, C, D, F, G) DIG-labeled anti-sense cRNA probes were used. (B, E) DIG-labeled sense cRNA probes were used. Seven-microgram sections of bovine placentome were hybridized with each probes. Scale bar = 100 μ m on the (A, B, D, E) and 20 μ m on the (C, F, G).

These configurations indicate common features in bPRPs/ bPLs. In particular, the configuration of the cysteine residue in bPRP-VIII coincided exactly with that of bPRP-III in the mature sequence region. The cysteine residue in bPRP-IX was also the same as that in bPRP-I, -II, and -IV in the mature sequence region. Therefore, both bPRP-VIII and -IX may have three disulfide bonds similar to those of other bPRPs, except for bPRP-VII [6].

The primary mRNA expression of *bPRP-VIII* and *-IX* was observed in the binucleate cells (Fig. 7). Both genes could independently produce mature recombinant proteins in the mammalian cell expression system (Fig. 9). The binucleate cells may have produced the bPRP-VIII and -IX proteins simultaneously because *bPRP-VIII* and *-IX* mRNA expressed in the binucleate cells, and HEK293 cells translated these mRNA to each protein individually. Binucleate cells are also primary expression cells for *bPRP-I* and *bPL* [27,28] and may have a specific function for implantation in the fetomaternal interface [11,29]. *bPRP-VIII* mRNA was also expressed in the extra-embryonic membrane just after the implantation period in the present study (Day 27 of gestation) (Fig. 8). bPRP-VIII may also be related to the

implantation process like bPRP-I and -VII since *bPRP-I* was expressed during early gestation. Compare to these expression, no *bPRP-IX* was present in the extra-embryonic membrane on Day 27 of gestation (Fig. 8). This mRNA expression pattern implies that bPRP-IX has a different role from *bPRP-I* and *bPL* in placental formation [6]. *bPRP-IX* mRNA was also detected in the trinucleate cells. A trinucleate cell is generated by migration of a binucleate cell to an endometrial epithelial cell with the progress of the pregnancy [30]. bPRP-IX may be a necessary molecule during middle to late gestation. Although both bPRP-VIII and -IX may be necessary molecules for gestation, *in situ* hybridization demonstrated that both genes were primarily expressed in binucleate and trinucleate cells. This means both genes derive from fetal side origin, but both were stably contained in the caruncular area throughout gestation, except bPRP-IX in early gestation. There are two potential causes of this result. One is contamination after separation. A second possibility is cell migration [30]. The present study and our previous study suggest that binucleate and trinucleate cells may migrate deeply into the endometrium after implantation and maintain their functions, which may be related to immu-

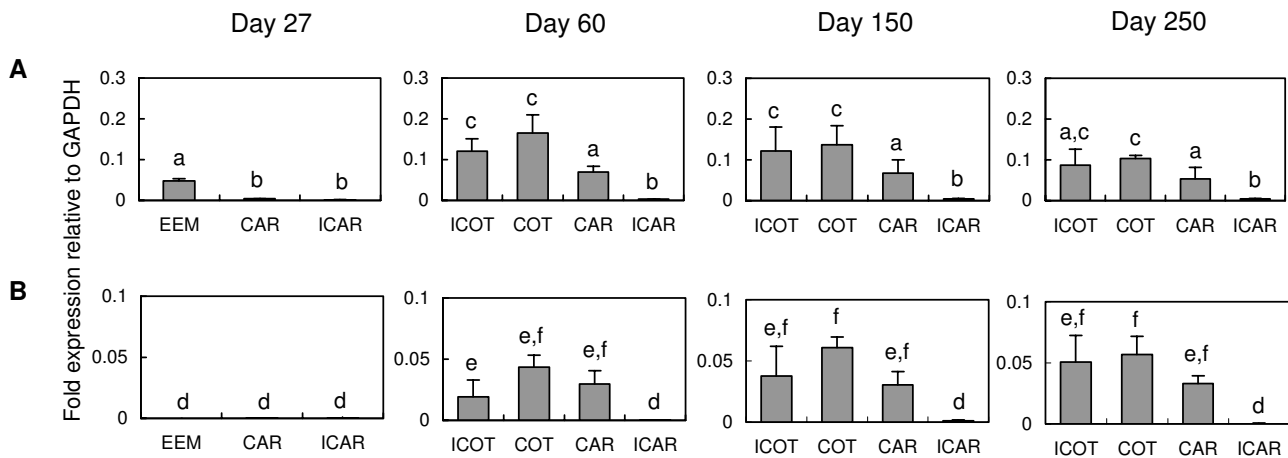


Figure 8
Quantitative real-time RT-PCR analysis of (A) bPRP-VIII and (B) bPRP-IX mRNA in bovine placenta. The total RNA was extracted from cotyledons containing extra-embryonic membrane (EEM), cotyledonary placenta (COT), intercotyledonary fetal membrane (ICOT), caruncular placenta (CAR), and intercaruncular endometrium (ICAR) on Day 27, Day 60, Day 150, and Day 250 of gestation. The expressions of these mRNAs were normalized to the expression of GAPDH measured in the same RNA preparation. Values are means \pm SD. Values with different letters are significantly different ($P < 0.05$).

nomodulation or stress during bovine gestation [6,7,30,31]. The molecular size of the recombinant proteins completely differed between bPRP-VIII and -IX. Transcribed bPRP-VIII protein is of relatively smaller size in the known bPRP family members, and the bPRP-IX protein is among the largest members [6,32,33]. The sugar chain addition in post-translational modification will give large effect in the difference of both molecular weights. It was found that the predicted 3D structure differed clearly in both molecules, because configuration of disulfide bonds and sugar chain addition dynamics are different. The tertiary structure of bPRP-VIII and -IX are similar to that of hPRL, so they may share the same receptor. However there is no information and the other evidence may not support this speculation. Only the *bPRP-I* gene has been known to produce protein in the placenta, however, the protein bound to the alpha2-macroglobulin [32], so it might have a biological function in paracrine status. Since all other bPRPs have a similar characteristic, it is difficult to say bPRPs are hormones secreted into circulation from the placenta, such as bPRL and bPL. It was possible to predict a part of their functions from the molecular weight, 3D structure, and post-translational modification. bPRP-VIII and -IX proteins may have different functions, since their temporal and special expressions were different. Various difficulties remain for understanding PRP functions, however, many of the same genes and molecules are expressed in bovine placenta as in rodent placenta [7].

Recently, bovine genome projects have addressed various shotgun sequence data and five known *bPRPs*, *bPL*, and *bPRL*, a total of seven prolactin related genes, hit in the NCBI genome database. They are located in *Bos taurus* chromosomes 12 and/or 23. *bPRP-III*, *-VII* and *bPL* were detected on chromosome 23 with *bPRL*. On the other hand, *bPRP-I*, *-V*, and *-VI* are placed on chromosome 12. Rodents such as mice and rats also have various *PLPs* [7,34], and they have been clustered on one chromosome, number 13 in mice and number 17 in rats [34,35]. *bPRP-III* and *-VII* were in comparatively close positions on the phylogenetic tree in Fig. 4, and they are clustered in the same chromosome 23. *bPRP-VIII* may be placed on chromosome 23 because *bPRP-VIII* was close to *bPRP-III* or *-VII* on the phylogenetic tree, although the bootstrap value is low between *bPRP-VIII* and *bPRP-III/-VII*. *bPRP-I*, *-V*, and *-VI*, however, were comparatively close on the phylogenetic tree in Fig. 4; these genes were clustered on chromosome 12. *bPRP-IX* may be placed on chromosome 12 because *bPRP-IX* was close to *bPRP-I* on the phylogenetic tree. The actual configuration of the *bPRP-VIII* and *-IX* genome will be addressed in the future. Therefore, prolactin-related genes in mammals may have similar roles for placental function, but they may evolve through different phylogenetic processes, may be two separate pathways. For example, GH and PRL functions are different in various species, namely, GH uses the PRL receptor in some species, but not in others [7,36].

In conclusion, we identified two new members of *bPRPs*, *bPRP-VIII* and *-IX*. *bPRP-VIII* was expressed in binucleate

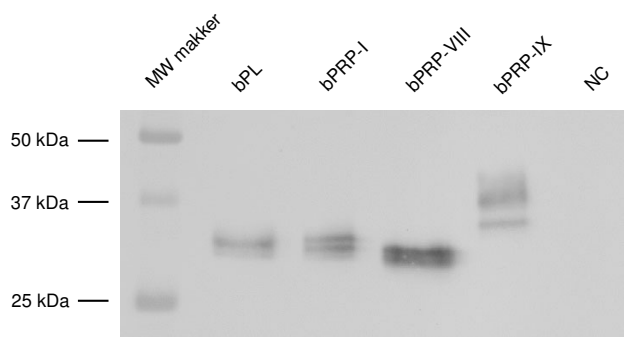


Figure 9
Production of recombinant bPRP-VIII, bPRP-IX, bPRP-I, and bPL proteins. Conditioned media from HEK 293 cells transiently transfected with each gene were collected, and the proteins (10 µg) were loaded in separate lanes. The proteins were separated by SDS PAGE and specific proteins were detected by Western blot analysis using an anti-FLAG tag. MW Marker: molecular weight marker. NC: Negative control (transfected vector only).

cells in bovine trophoblast tissue and placentome. The expression appeared as well in *bPRP-I*, *-VII*, and *bPL*, and it expressed from the implantation period to late in gestation. *bPRP-IX* was expressed in binucleate cells and trinucleate cells, and was expressed somewhat late in gestation compared to other PRPs. These data indicate that various PRP genes in bovine placenta have coordination roles for gestation as evidenced in rodents.

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References

1. Dietz AB, Georges M, Threadgill DW, Womack JE, Schuler LA: Somatic cell mapping, polymorphism, and linkage analysis of bovine prolactin-related proteins and placental lactogen. *Genomics* 1992, **14**:137-143.
2. Schuler LA, Kessler MA, Tanaka M, Nakashima K: **Nomenclature clarification for the bovine placental prolactin-related hormones.** *Endocrinology* 1991, **126**:2057.
3. Ushizawa K, Herath CB, Kaneyama K, Shiojima S, Hirasawa A, Takahashi T, Imai K, Ochiai K, Tokunaga T, Tsunoda Y, Tsujimoto G, Hashizume K: **cDNA microarray analysis of bovine embryo gene expression profiles during the pre-implantation period.** *Reprod Biol Endocrinol* 2004, **2**:77.
4. Kessler MA, Duello TM, Schuler LA: **Expression of prolactin-related hormones in the early bovine conceptus, and potential for paracrine effect on the endometrium.** *Endocrinology* 1991, **129**:1885-1895.

5. Yamada O, Todoroki J, Kizaki K, Takahashi T, Imai K, Patel OV, Schuler LA, Hashizume K: **Expression of prolactin-related protein I at the fetomaternal interface during the implantation period in cows.** *Reproduction* 2002, **124**:427-437.
6. Ushizawa K, Kaneyama K, Takahashi T, Tokunaga T, Tsunoda Y, Hashizume K: **Cloning and expression of a new member of prolactin-related protein in bovine placenta: bovine prolactin-related protein-VII.** *Biochem Biophys Res Commun* 2005, **326**:435-441.
7. Soares MJ: **The prolactin and growth hormone families: Pregnancy-specific hormones/cytokines at the maternal-fetal interface.** *Reprod Biol Endocrinol* 2004, **2**:51.
8. Muller H, Liu B, Croy BA, Head JR, Hunt JS, Dai G, Soares MJ: **Uterine natural killer cells are targets for a trophoblast cell-specific cytokine, prolactin-like protein-A.** *Endocrinology* 1999, **140**:2711-2720.
9. Ain R, Tash JS, Soares MJ: **Prolactin-like protein-A is a functional modulator of natural killer cells at the maternal-fetal interface.** *Mol Cell Endocrinol* 2003, **204**:65-74.
10. Lin J, Linzer DIH: **Induction of megakaryocyte differentiation by a novel pregnancy-specific hormone.** *J Biol Chem* 1999, **274**:21485-21489.
11. Bittorf T, Jaster R, Soares MJ, Seiler J, Brock J, Friese K, Muller H: **Induction of erythroid proliferation and differentiation by a trophoblast-specific cytokine involves activation of the JAK/STAT pathway.** *J Mol Endocrinol* 2000, **25**:253-262.
12. Bhattacharyya S, Lin J, Linzer DIH: **Reactivation of a hematopoietic endocrine program of pregnancy contributes to recovery from thrombocytopenia.** *Mol Endocrinol* 2002, **16**:1386-1393.
13. Schuler LA, Hurley WL: **Molecular cloning of a prolactin-related mRNA expressed in bovine placenta.** *Proc Natl Acad Sci USA* 1987, **84**:5650-5654.
14. Kessler MA, Milosavljevic M, Zieler CG, Schuler LA: **A subfamily of bovine prolactin-related transcripts distinct from placental lactogen in the fetal placenta.** *Biochemistry* 1989, **28**:5154-5161.
15. Yamakawa M, Tanaka M, Koyama M, Kagesato Y, Watahiki M, Yamamoto M, Nakashima K: **Expression of new members of the prolactin growth hormone gene family in bovine placenta. Isolation and characterization of two prolactin-like cDNA clones.** *J Biol Chem* 1990, **265**:8915-8920.
16. Tanaka M, Yamakawa M, Watahiki M, Yamamoto M, Nakashima K: **Isolation of a novel prolactin-like cDNA clone from bovine placenta: occurrence of new family members.** *Biochim Biophys Acta* 1989, **1008**:193-197.
17. Tanaka M, Minoura H, Ushiro H, Nakashima K: **A novel cDNA clone encoding a prolactin-like protein that lacks the two C-terminal cysteine residues isolated from bovine placenta.** *Biochim Biophys Acta* 1991, **1088**:385-389.
18. Saitou N, Nei N: **A neighbor-joining method: a new method for constructing phylogenetic tree.** *Mol Biol Evol* 1987, **44**:406-425.
19. Page RDM: **TREEVIEW: An application to display phylogenetic trees on personal computers.** *Comput Appl Biosci* 1996, **12**:357-358.
20. Ogata K, Umeyama H: **An automatic homology modeling method consisting of database searches and simulated annealing.** *J Mol Graphics Mod* 2000, **18**:258-272.
21. Bernstein HJ: **Recent changes to RasMol, recombining the variants.** *Trends Biochem Sci* 2000, **25**:453-455.
22. Herath CB, Shiojima S, Ishiwata H, Katsuma S, Kadowaki T, Ushizawa K, Imai K, Takahashi T, Hirasawa A, Tsujimoto G, Hashizume K: **Pregnancy-associated changes in genome-wide gene expression profiles in the liver of cow throughout pregnancy.** *Biochem Biophys Res Commun* 2004, **313**:666-680.
23. Kizaki K, Yamada O, Nakano H, Takahashi T, Yamauchi N, Imai K, Hashizume K: **Cloning and localization of heparanase in bovine placenta.** *Placenta* 2003, **24**:424-430.
24. Kizaki K, Nakano H, Nakano H, Takahashi T, Imai K, Hashizume K: **Expression of heparanase mRNA in bovine placenta during gestation.** *Reproduction* 2001, **121**:573-580.
25. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature* 1970, **227**:680-685.
26. Towbin H, Staehelin T, Gordon J: **Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.** *Proc Natl Acad Sci USA* 1979, **76**:4350-4354.

27. Milosavljevic M, Duello TM, Schuler LA: **In situ localization of two prolactin-related messenger ribonucleic acids to binucleate cells of bovine placentomes.** *Endocrinology* 1989, **125**:883-889.
28. Nakano H, Takahashi T, Imai K, Hashizume K: **Expression of placental lactogen and cytokeratin in bovine placental binucleate cells in culture.** *Cell Tissue Res* 2001, **303**:263-270.
29. Anthony RV, Liang R, Kayl EP, Pratt SL: **The growth hormone/prolactin gene family in ruminant placentae.** *J Reprod Fertil Suppl* 1995, **49**:83-95.
30. Wooding FBP, Beckers JF: **Trinucleate cells and the ultrastructural localisation of bovine placental lactogen.** *Cell Tissue Res* 1987, **247**:667-673.
31. Ain R, Dai G, Dunmore JH, Godwin AR, Soares MJ: **A prolactin family paralog regulates reproductive adaptations to a physiological stressor.** *Proc Nat Acad Sci USA* 2004, **101**:16543-16548.
32. Kessler MA, Schuler LA: **Purification and properties of placental prolactin-related protein-I.** *Placenta* 1997, **18**:29-36.
33. Takahashi T, Aso H, Hashizume K: **Immunological and biological activities of bovine placental lactogen in placental explant culture.** *J Reprod Dev* 2001, **47**:63-67.
34. Wiemers DO, Shao LJ, Ain R, Dai G, Soares MJ: **The mouse prolactin gene family locus.** *Endocrinology* 2003, **144**:313-25.
35. Öztürk A, Fresnoza A, Savoie A, Duckworth HW, Duckworth ML: **Defining regulatory regions in the rat prolactin gene family locus using a large P1 genomic clone.** *Endocrinology* 2003, **144**:4742-4754.
36. Talamantes F, Ogren L, Markoff E, Woodard S, Madrid J: **Phylogenetic distribution, regulation of secretion, and prolactin-like effects of placental lactogens.** *Fed Proc* 1980, **39**:2582-2587.

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