

Molecular characterization of the bovine *GHRL* gene

(Short Communication)

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Abstract

Bovine ghrelin, a 27 amino acid peptide, has been identified in oxyntic glands of the abomasum. It is an endogenous ligand for growth hormone secretagogue receptor and stimulates food intake and growth hormone secretion. The bovine *GHRL* gene was completely sequenced and consists of five exons and four introns. Like mouse and human *GHRL* genes, we found that the bovine *GHRL* gene also contains a first non-coding exon of 21 bp. The bovine *GHRL* gene codes for 116 amino acid peptide named preproghrelin which contains the ghrelin peptide and another peptide similar to obestatin. Sequence analysis revealed eight polymorphisms, which are located in the non-coding sequence of the gene.

Keywords: *GHRL*, ghrelin, cattle, polymorphism, sequence

Zusammenfassung

Molekulare Charakterisierung des bovinen *GHRL* Gens (Kurzmitteilung)

Bovines Ghrelin, ein Peptid mit 27 Aminosäuren, ist in den oxyntischen Drüsen des Labmagens identifiziert worden. Es ist ein endogener Ligand des GHS-Rezeptor (Growth Hormone Secretagogue Receptor) und stimuliert die Futteraufnahme und Wachstumshormonsekretion. Das bovine *GHRL* Gen wurde vollständig sequenziert und besteht aus fünf Exons und vier Introns. Wie beim humanen und murinen Gen weist auch das bovine *GHRL* ein nicht kodierendes erstes Exon von 21 bp auf. Das bovine *GHRL* kodiert ein 116 Aminosäuren langes Prepro-Ghrelin, das das Ghrelin-Peptid und ein Peptide ähnlich dem Obestatin einhält. Sequenzanalysen zeigen in der nicht kodierenden Sequenz des Gens acht Polymorphismen an.

Schlüsselwörter: *GHRL*, Ghrelin, Rind, Polymorphismus, Sequenz

Introduction

Ghrelin, a growth hormone (GH)-releasing peptide, was first isolated from rat and human as an endogenous ligand for the growth hormone secretagogue receptor (KOJIMA *et al.* 1999). In cattle, cells immunostained for ghrelin are distributed in the area of the abomasum (HAYASHIDA *et al.* 2001). It has been suggested that this molecule is secreted from the stomach and circulates in the bloodstream to stimulate GH secretion in ruminants. Plasma ghrelin levels decrease 1 h after feeding and then return to prefeeding levels in cows (HAYASHIDA *et al.* 2001). Injection of bovine ghrelin at physiological

concentration resulted in elevated plasma GH concentrations and length of time spent eating was greater (WERTZ-LUTZ *et al.* 2006). During peak of lactation, plasma concentrations of ghrelin and GH are greater in samples from high genetic merit Holstein-Friesian cows (ROCHE *et al.* 2006). Recently, SHERMAN *et al.* (2008) found an A/G SNP in intron 3 of bovine *GHRL* gene. Also, five SNPs were detected in bovine *GHSR* gene (ZHANG *et al.* 2007).

Considering the effects of ghrelin on the growth hormone axis and the control of enteric nutrition, this study was conducted to characterize the genomic organization, DNA sequence and polymorphisms of the bovine *GHRL* gene.

Material and methods

Material

DNA extracted from semen straws of Limousin bulls ($n=10$), double-muscléd Belgian Blue bulls ($n=10$) and Holstein bulls ($n=10$) were included in the polymorphisms discovery study. Additionally, DNA from semen straws of Simmental bulls ($n=133$) and Holstein bulls ($n=127$) and from ear biopsy of Belgian Blue calves ($n=86$) were studied for observed allele and genotype frequencies.

Full-length ghrelin cDNA sequence

Total RNA was extracted from a Belgian Blue bull abomasum by TriPure Isolation Reagent (Roche Applied Science, USA). In order to obtain the full-length ghrelin cDNA sequence, rapid amplification of cDNA ends (RACE) was carried out with de BD Smart RACE cDNA Amplification Kit (Clontech, CA, USA). 5'- and 3'-RACE-Ready cDNA were synthesized according to manufacturer's instructions. Gene-specific primers were designed based on partial sequences of bovine ghrelin CDS (accession AY455980 and AY455979).

For 5'RACE, the first PCR was performed using 5'RACE Ready cDNA as a template with a combination of the universal primer mix provided by the kit and primer P1r CTGAGG GTGGGAGAACGGACA. All PCR products were amplified using a regular PCR with Taq DNA Polymerase (GE Healthcare, USA). The annealing temperature was 54°C. The nested PCR was then performed using an aliquot of the first PCR reaction mixture as a template with a combination of the nested universal primer provided by the kit and primer P2r GCCTTCCAGGGTCCG. PCR conditions were identical to those of the first PCR.

For 3'RACE, the first PCR was performed using 3'RACE Ready cDNA as a template with a combination of the universal primer mix and primer P3f ATGCCCGCCCCGTGGA. PCR conditions were the same as described above. The nested PCR was then performed with primer P4f CAACGCCCCCTTAAACAT and the annealing temperature was 55°C.

Both amplified products were purified using Montage PCR filter units (Millipore, USA) and directly sequenced by using BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Sequencing reactions were resolved on an ABI Prism 3130 automated capillary DNA sequencer (Applied Biosystems, USA). Full-length cDNA sequence was compared to the draft of the bovine genome sequence by BLASTN to identify exons.

Polymorphism research

The five exons were PCR amplified from Holstein ($n=10$), Limousin ($n=10$) and Belgian Blue ($n=10$) bulls. The five sets of primers are given Table 1. The PCR conditions were the same as described above and the annealing temperatures are presented in the Table 1. After purification using Montage PCR filter units (Millipore, USA), the amplification products were directly sequenced as described above.

Genotyping

Given the proximal localization of the five SNPs g.267, g.271, g.290, g.326 and g.327, animals were genotyped by direct sequencing of the first exon as described above.

The three other SNPs (g.993, g.4491 and g.4644) were genotyped by Single-Base Extension. Two sets of primers, P10f-P10r and P11f-P11r, were designed to amplify flanking genomic DNA. PCR conditions were the same as described above and the annealing temperatures are presented in Table 1. The amplification products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Germany). The purified products then were used as templates for the primer extension reaction using the SnaPshot Multiplex Kit (Applied Biosystems, USA). The designed primers for this reaction are GACTGACTGACTCTCTCCGAGAAGCA (g.993), GACTGACTGACTGACTGATCTTAAGTTCCCA (g.4491) and GACTACCAACCACCTGTCC (g.4644). The reaction mixture was treated with calf intestinal alkaline phosphatase to remove unincorporated ddNTPs. The samples containing extension products and Genescan 120 LIZ size standard solution were added to HiDi formamide according to the recommendation of the manufacturer. The mixture was incubated at 95°C for 5 min, followed by 5 min on ice, the electrophoresis was performed on an ABI Prism 3130 Genetic Analyzer. The results finally were analyzed using GeneScan Analysis Software v3.7 (Applied Biosystems, USA).

Table 1

Primer sequences designed from the bovine *GHRL* gene (acc. no. AM691749) and used in amplification of genomic fragments

Primersequenzen abgeleitet vom bovinen GHRL Gen (acc. no. AM69749), die zur Amplifikation genomischer Abschnitte genutzt wurden

Primer	Sequence	Ta (°C)	PI (bp)	Use
P6f	TCCCGCCAAGCCGAGG	60	337	Exon 1 sequencing
P6r	TGTTGCCGTGAGTTAGACCC			
P7f	AGCCACCATCTCTCTGCC	58	345	Exon 2 sequencing
P7r	AATCCCGCTCTCTCC			
P8f	ATGCCGCCCCGTGGA	58	513	Exon 3 sequencing
P8r	TGCTGTTTGATGTCCTTGA			
P9f	TGACCTTGACAATTCTG	58	312	Exon 4 sequencing
P9r	GGACCAATCCCCACG			
P10f	TCTCGCCGTTACAGACA	56	341	Exon 5 sequencing
P10r	TCCCGGAAGCCAGGTGAGA			
P11f	ATGCCGCCCCGTGGA	57	405	Primary PCR for SBE
P11r	TCTGCACCTTCGCCTGACTT			

Ta annealing-temperature, PI Product length

Results and discussion

The *bGHRL* cDNA is 532 bp long, consisting of 47 bp 5'-noncoding region, 351 bp coding region and 134 bp 3'-noncoding region. The complete *bGHRL* sequence has been submitted to the EMBL nucleotide database (acc. no. AM691749). The genomic sequence analysis revealed that the *GHRL* gene consists of five exons and four introns (Figure 1), the intron-exon boundary strictly follows the GT-AG rule. The first exon contains only 21 bp and is presented at the non-coding region of cDNA. Similar results were observed in mouse and human *GHRL* genes (TANAKA *et al.* 2001, NAKAI *et al.* 2004). We demonstrated that bovine *GHRL* gene exons 2, 3, 4 and 5 are respectively 134, 114, 109 and 154 bp long. A TATA box-like sequence is localized 29 bp upstream of the transcription start site of the bovine *GHRL* gene. This characterization could be useful for ongoing annotation of the bovine genome.

The preproghrelin encoded by bovine *GHRL* gene contains 116 amino acids and has 96, 78, 75, 75 and 71% identity respectively with ovine, swine, rat, mouse and human preproghrelin. In the preproghrelin sequence, the ghrelin peptide corresponds to 27 residues (from residue 24 to 50). A sequence of 23 residues (from residue 75 to 97) similar to human obestatin was also detected in the preproghrelin sequence.

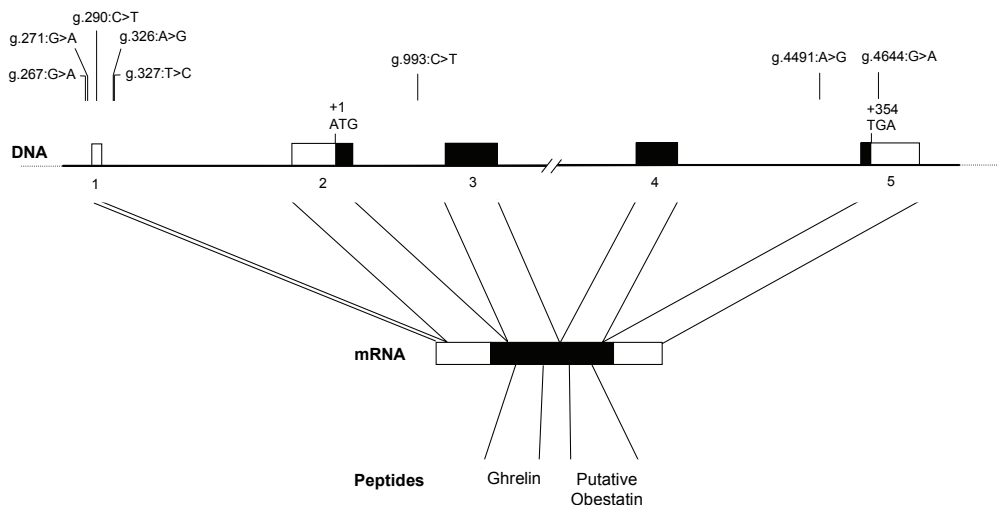


Figure 1

Genomic structure of the bovine *GHRL* gene. Solid boxes correspond to translated exons. Open boxes correspond to untranslated regions. The eight described SNPs are located at the top. Sequences coding for ghrelin and putative obestatin peptides are also located on the mRNA.

Genomische Struktur des bovinen GHRL Gens. Gefüllte Boxen sind translatierte Exons, offene Boxen sind untranslatierte Regionen. Die acht identifizierten SNPs sind oben angegeben. Sequenzen, die Ghrelin und Obestatin kodieren, sind in der mRNA markiert.

It has been predicted that all studied ruminant species have only the 27 amino acid form of the mature ghrelin peptide. The first splicing acceptor site within intron 2 has been lost as a result of a transition event (adenine to thymine) (DICKIN *et al.* 2004), and the present study supports this finding. By BLAST searching the GenBank database, another cDNA

(acc. no. BC148027) was detected and this mRNA was extracted from bovine placenta. This sequence presents a supplementary exon between exons 4 and 5. Other splicing variants were also reported in human and mouse. Therefore, *GHRL* gene could be differently expressed in bovine tissues, suggesting other potential biological functions of the preproghrelin gene by alternative splicing.

WAJNRAJCH *et al.* (2000) mapped human *GHRL* gene on HSA 3p26-p25. An alignment on Btau_4.0 assembly permit to locate the bovine *GHRL* gene on BTA 22. This location agrees totally with comparative mapping data between cattle and man since BTA 22 corresponds to part of human chromosome 3 (HAYES 1995).

Eight single nucleotide polymorphisms were identified and located in non-coding regions (Figure 1); all of them are transversion polymorphisms.

Allele and genotype frequencies of these eight polymorphisms are listed in Table 2. We observed that the identified polymorphisms were rare in the Holstein population but not in the Belgian Blue and Simmental populations. The g.4491 variant was previously detected (SHERMAN *et al.* 2008). The g.290T variant was not present in studied Holstein and Simmental populations. Only three Belgian Blue calves heterozygote for g.290C>T were found. Considering all visually inspected sequencing traces, we speculate that g.326 and g.327 could be a dinucleotide polymorphism (g.326AT>GC).

Given their locations, it would be interesting to perform further experiments to determine if g.267A, g.271A, g.290T, g.326G or g.327C variants have an effect on the transcription rate of the bovine *GHRL* gene.

Table 2

Allele frequencies observed for each SNP within the bovine *GHRL* gene in the studied Holstein, Belgian Blue and Simmental groups

Relative Häufigkeit von Allelen für jeden SNP im bovinen GHRL Gen bei Holstein, Weiß-blaue Belgier und Simmentaler

SNP	Allele		Holstein		Belgian Blue		Simmental	
	M	m	M	m	M	m	M	m
g.267	G	A	0.980	0.020	0.811	0.189	0.759	0.241
g.271	G	A	0.980	0.020	0.837	0.163	0.846	0.154
g.290	C	T	1.000	0.000	0.982	0.018	1.000	0.000
g.326	A	G	0.980	0.020	0.774	0.226	0.767	0.233
g.327	T	C	0.980	0.020	0.785	0.215	0.767	0.233
g.993	C	T	0.949	0.051	0.860	0.140	0.989	0.011
g.4491	A	G	0.972	0.028	0.814	0.186	0.756	0.244
g.4644	G	A	0.992	0.008	0.948	0.052	1.000	0.000

Location of the SNPs refers to the genomic sequence of 5030 bp (AM691749), M major allele, m minor allele

In conclusion, this study characterizes the bovine *GHRL* gene. Eight polymorphisms were detected. These eight SNPs are present in different cattle breeds and could be used for association studies.

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