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)


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-muscled 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 ATGCCCCCGCCGTCGA. PCR conditions were the same as described above. The nested PCR was then performed with primer P4f CAAAGCCCGCTTTAACAT 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 Produkt 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 GACTGACTGACTCTCTCGGAGAAGCA (g.993), GACTGACTGACTGACTGATCTTTAAGTTTCCCCA (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>
<thead>
<tr>
<th>Primer Sequence Ta (°C) Pl (bp) Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6f TCCGCGAAGGCGGAGG 60 337 Exon 1 sequencing</td>
</tr>
<tr>
<td>P6r TGTTGCGGAGAGTTAGACCC 60 337 Exon 2 sequencing</td>
</tr>
<tr>
<td>P7f AGCCACCATCTCTCGGC 58 345 Exon 3 sequencing</td>
</tr>
<tr>
<td>P7r AATCCGGCTCTCTGC 58 345 Exon 4 sequencing</td>
</tr>
<tr>
<td>P8f ATGCCCGCCCCGTGG 58 345 Exon 5 sequencing</td>
</tr>
<tr>
<td>P8r TGCTGTTTGTGCTCTTGA 58 345 Exon 5 sequencing</td>
</tr>
<tr>
<td>P9f TGACCCCTGAATTTCTGT 58 345 Exon 5 sequencing</td>
</tr>
<tr>
<td>P9r GGACCAGATCCCGCACG 58 345 Exon 5 sequencing</td>
</tr>
<tr>
<td>P10f TCTCGCCGTTACAGCACA 56 341 Primary PCR for SBE</td>
</tr>
<tr>
<td>P10r TCCGCGAAGGCGGAGG 56 341 Primary PCR for SBE</td>
</tr>
<tr>
<td>P11f ATGCCGCCCCGTGG 57 405 Primary PCR for SBE</td>
</tr>
</tbody>
</table>

Ta annealing-temperature, Pl 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.


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, \textit{GHRL} gene could be differently expressed in bovine tissues, suggesting other potential biological functions of the preproghrelin gene by alternative splicing.

WAJNRAJCH \textit{et al.} (2000) mapped human \textit{GHRL} gene on HSA 3p26-p25. An alignment on Btau_4.0 assembly permit to locate the bovine \textit{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 \textit{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 \textit{GHRL} gene.

Table 2

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>Holstein</th>
<th>Belgian Blue</th>
<th>Simmental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>m</td>
<td>M</td>
<td>m</td>
</tr>
<tr>
<td>g.267</td>
<td>A</td>
<td>0.980</td>
<td>0.020</td>
<td>0.811</td>
</tr>
<tr>
<td>g.271</td>
<td>A</td>
<td>0.980</td>
<td>0.020</td>
<td>0.837</td>
</tr>
<tr>
<td>g.290</td>
<td>C</td>
<td>1.000</td>
<td>0.000</td>
<td>0.982</td>
</tr>
<tr>
<td>g.326</td>
<td>A</td>
<td>0.980</td>
<td>0.020</td>
<td>0.774</td>
</tr>
<tr>
<td>g.327</td>
<td>T</td>
<td>0.980</td>
<td>0.020</td>
<td>0.785</td>
</tr>
<tr>
<td>g.993</td>
<td>C</td>
<td>0.949</td>
<td>0.051</td>
<td>0.860</td>
</tr>
<tr>
<td>g.4491</td>
<td>A</td>
<td>0.972</td>
<td>0.028</td>
<td>0.814</td>
</tr>
<tr>
<td>g.4644</td>
<td>G</td>
<td>0.992</td>
<td>0.008</td>
<td>0.948</td>
</tr>
</tbody>
</table>

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 \textit{GHRL} gene. Eight polymorphisms were detected. These eight SNPs are present in different cattle breeds and could be used for association studies.

**Acknowledgements**

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