Mapping and polymorphism of bovine ghrelin gene

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Introduction

Ghrelin, a growth hormone(GH)-releasing peptide, was isolated from rat and human stomach as an endogenous ligand for the growth hormone secretagogue receptor in 1999. Ghrelin has been identified in bovine oxyntic glands of the abomasum. Rat ghrelin stimulates GH release from bovine pituitary cells *in vitro* and *in vivo*. It circulates at considerable plasma concentrations in cattle. It has been reported that plasma ghrelin levels decrease 1h after feeding and then return to the prefeeding levels in cow. This peptide may function in the regulation of feeding or energy balance in ruminants.

Aim

Given the influences of ghrelin on the growth hormone axis and the regulation of feeding, this work aims at the study of the bovine ghrelin gene in order to evaluate the potential involvment of ghrelin in genetic variation for growth performances or milk yield.

Results

Sequencing and identification of polymorphisms

Principle: Total RNA was extracted from bovine abomasum by TriPure Isolation Reagent (Roche Applied Science). Complete ghrelin mRNA was sequenced by rapid amplification of cDNA ends (BD Smart™ RACE cDNA Amplification Kit, Clontech). Complete and partial sequences of unknown introns were determined by genome walker (Universal GenomeWalker Kit, Clontech). Primers were designed to sequence the exons 2, 3, 4 and 5 and introns II and IV. Ten Belgian White Blue bulls, ten Holsteins bulls and ten Limousin bulls were screened for polymorphisms.

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1    CACCTCCGCC  CGGAACCCCA  GGTCCATCTG  CCTCCAGCCA  GGGAGCCATG
51   CCCGCCCCGT  GGACCATCTG  CAGCCTGCTG  CTGCTCAGCG  TGCTCTGCAT
101  GGACTTGGCC  ATGGCGGGCT  CCAGCTTTCT  GAGCCCCGAA  CATCAGAAAC
151  TGCAGAGAAA  GGAAGCTAAG  AAGCCATCAG  GCAGACTGAA  GCCCCGGACC
201  CTGGAAGGCC  AGTTTGACCC  GGAGGTGGCA  AGTCAGGCGG  AAGGTCCAGA
251  GGACGAGCTG  GAAATCCCGT  TCAACGCCCC  CTTTAACATT  GGGATCAAGC
301  TAGCAGGGGC  TCAGTCCCTC  CAGCATGGCC  AGACGTTGGG  GAAGTTTCTT
351  CAGGACATCC  TTTGGGAAGA  AGCTGAAGAA  ACCCTGGCTA  ACGAGTGAGT
401  GGCCCTGGGA  CCAACCACCT  GTCCGTTCTC  CCACCCTCAG  AAGCTCTCAC
451  CTGGCTTCCG  GGACACTTCC  GAGACCACGT  GGGGCTCTGA  GGGGTACTAG
501  AGTAGGCAGT  GAATAAATGC  TCAGATGGAT  GCAAAAAAA
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Figure 1. Complete mRNA sequence. The sequence contains five exons (each colour). The sequence between nt 48 and nt 398 represent the CDS. The poly(A) signal sequence is at 511 nt and followed by 14 nucleotides sequence, a CA sequence and the poly(A) tail.

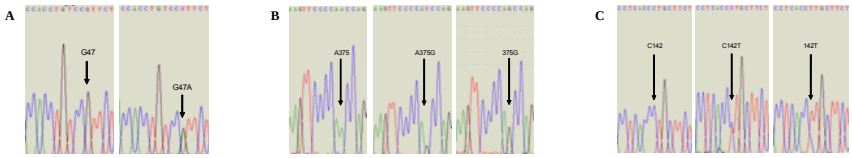

Figure 2. Sequencing electropherograms showing single nucleotide polymorphism detection. (A) Partials sequences with Mut 1 at nucleotide 47 of exon 5 (G47A). (B) Partials sequences with Mut 2 at nucleotide 375 of intron IV (A375G). (C) Partials sequences with Mut 4 at nucleotide 142 of intron II (C142T).

The bovine ghrelin gene contains 5 exons and 4 introns with a short noncoding first exon of 21 bp similar to mouse and human ghrelin gene. The screening for polymorphisms revealed a total of 3 SNPs.

Mapping of bovine ghrelin gene

Using a radiation hybrid panel, the gene was mapped to chromosome 22 near microsatellite markers UWCA49, BM4102, BMS1932, BM2613 and URB035 with good LOD Score. Some studies detected different QTLs near these markers. Boichard *et al.* (2003) detected a QTL for milk fat percent near UWCA49 and Ashwell *et al.* (2004) reported QTLs for milk protein percent and somatic cell score near BM4102.

In order to evaluate the potential involvment of ghrelin in genetic variation for milk fat percent, milk protein percent and somatic cell score, an association study between SNPs on ghrelin gene and these traits could be performed in a cattle population.

Genotyping by single-base extension (SBE) and electrophoresis

Principle: For genotyping of polymorphic sites, amplifying and extension primers were designed for single-base extension (SBE). Primer extension reactions were performed with the SNaPshot Multiplex Kit (Applied Biosystems). To remove unincorporated ddNTPs, one unit of CIP (Calf Intestinal alkaline Phosphatase) was added to the reaction mixture, and the mixture was incubated at 37 °C for 1 hour, followed by 15 min at 72 °C for enzyme inactivation. The DNA samples, containing extension products, and Genescan 120 LIZ size standard solution was added to Hi-Di formamide (Applied Biosystems) according to the recommendations 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 3100 Genetic Analyzer. The results were analyzed using the program of GeneScan Analysis Software v3.7 (Applied Biosystems).

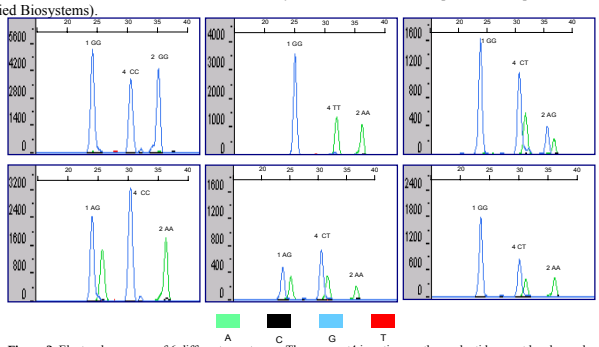

Figure 3. Electropherograms of 6 different genotypes. The snapmut4 is antisense, the nucleotides must be changed : A → T and G →C. The X-axis indicates the length of the DNA fragment. The Y-axis indicates relative fluorescence units (RFU).

Table 1. Genotype and allele frequencies observed in two different bovine breeds. The Hosltein group include 104 commercially available bulls used in Walloon Region and the Belgian White Blue group include 86 bulls from a bull-fattening entreprise.

			Holstein		Belgian White Blue	
			n	frequence	n	frequence
Mut 1	Genotypic classis	GG	102	98,1%	77	89,5%
		AG	2	1,9%	9	10,5%
		AA	0	0,0%	0	0,0%
	Allele	G		99,0%		94,8%
		A		1,0%		5,2%
Mut 2	Genotypic classis	AA	97	93,3%	56	65,1%
		AG	7	6,7%	28	32,6%
		GG	0	0,0%	2	2,3%
	Allele	A		96,6%		81,4%
		G		3,4%		18,6%
Mut 4	Genotypic classis	CC	94	90,4%	63	73,3%
		CT	10	9,6%	22	25,6%
		TT	0	0,0%	1	1,2%
	Allele	C		95,2%		86,0%
		T		4,8%		14,0%

Genotype and allele frequencies seem to be different between the two breeds. Neither Mut 1 AA, Mut 2 GG nor Mut 4 TT genotypes were found in the studied Holstein population. The statistical analysis was performed on the Holstein population because the Belgian White Blue bulls were still in fattening.

Statistical analysis

Statistical analysis was performed using the GLM procedure of SAS. The model used was a fixed model :

$$\mathbf{y = X\,b + e}$$

Where **y** = vector of estimated breeding value of bulls;

b = unknown vector of mean effect and regression coefficient;

X = known design matrix of fixed genotype effect, matrix linking **y** and **b**;

e = unknown vector of random residual effects.

The regression coefficient represented the gene substitution effect α. This model was solved using the following fixed model equations :

$$\mathbf{X'\,R^{-1}X\hat{b} = X'\,R^{-1}y \iff \hat{b} = (X'\,R^{-1}X)^{-1}X'\,R^{-1}y}$$

where $\mathbf{R^{-1}} = \mathbf{D} / \sigma^2_e$ where **D** = a diagonal matrix divided by the estimate of the residual variance σ^2_e . The diagonal element of **D**, representing the weight given to every bull, was computed as weight = reliability.

Table 2. Regression coefficient on the number of the allele and standard errors observed on 104 Holstein bulls

Trait	Mutation 1 G/A Allele A			Mutation 2 A/G Allele G			Mutation 4 C/T Allele T		
	α	SE		α	SE		α	SE	
Production									
Milk (kg)	30	240		-82	151		-66	123	
Fat (kg)	3,7	10,9		-10,6	6,7		-9,3	5,5	‡
Protein (kg)	-2,1	6,8		-2,7	4,2		-0,4	3,5	
Fat (%)	0,027	0,164		-0,106	0,102		-0,094	0,083	
Protein (%)	-0,046	0,075		-0,004	0,047		0,022	0,038	
Functional									
Somatic Cell Score	0,50	0,23	*	-0,04	0,20		0,05	0,14	
Herd Life	-0,16	0,16		0,11	0,12		-0,13	0,1	

‡ P < 0,10
* P < 0,05

Mut 1 is associated with an increase in somatic cell score so it wouldn't be interesting to use Mut 1 A allele through selection for this factor. Mut 4 show marginal association with fat milk. Finally, greater numbers of Holstein bulls with missing pattern would be helpful.

Conclusion

These results suggest that polymorphisms in bovine ghrelin gene are a promising new possibility to select for increased milk yield. However, these findings must be validated on a larger number of animals. Further genetic study is underway to investigate the ghrelin effet on performances in Belgian White Blue bulls.

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