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Full Length Research Paper

Identification and characterization of variants in the 5' flanking region of bovine growth hormone gene

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Sequence variations within the 5' flanking region of bovine growth hormone gene were identified from six bovine species raised in China. Cloned and sequenced amplified fragments revealed difference in length because of the insertion and deletion mutation. A total of thirty one variation sites were identified in this region within species and among species. Several new single nucleotide polymorphisms (SNPs) within bovine species were detected in the 5' flanking region with exception in swamp buffalo. Some important regulatory elements such as TATA box, CRE, NRE3, dPit1 and pPit1 were identified in the 5' flanking in six bovine species. The conservation of regulatory elements may be consistent with functional constraint during the course of evolution.

Key words: Bovine species, growth hormone gene, variation, regulatory element.

INTRODUCTION

Growth hormone (GH), the product of the GH gene, is a multifunctional hormone, produced in the vertebrate pituitary gland, which promotes the postnatal growth of skeletal and soft tissues. The bovine growth hormone (bGH) gene, mapped to chromosome 19 contains about 2856 nucleotides, mainly distributed in five exons and four introns (Gordon et al., 1983; Fries et al., 1993). During the last decade, extensive researches have shown a close relationship between polymorphism of bGH gene and production traits (Grochowska et al., 2001; Ge et al., 2003; Wu et al., 2005; Zhou et al., 2005; China et al., 2007; Pawar et al., 2007; Dario et al., 2008; Tatsuda et al., 2008; Ishida et al., 2010; Mullen et al., 2010; Matsuhashi et al., 2011). Therefore, it has been considered a candidate gene for production traits, such as milk production and carcass traits. The 5' flanking region of bGH gene contains repetitive regions and protein binding sites, such as the transcription initiation sites and transcription factor binding sites, which are associated with gene expression control.

Recent developments in molecular biotechnologies have opened the possibility of identifying and using genomic markers and multiple genes for the genetic improvement of livestock (Montaldo and Meza-Herrera, 1998; Dekkers, 2004; Margawati, 2012). This has provided opportunities to enhance response to selection, in particular for traits that are difficult to improve by conventional selection. The use of genetic markers or more effective of marker-assisted selection (MAS) for desired important traits would be more valuable and useful and even more efficient in important trait selection of superior livestock. Application of molecular biotechnology approaches will enable improvement in productivity, reduction in costs, enrichment of milk compositions and extension of shelf life products.

Bovine species are one of the most economically important domestic animals in China (Chang, 2009). It plays an important role in the economies of livestock sector. It provides food, or more specifically animal protein in human diets, income, employment and possibly foreign exchange. Our objective was to identify and characterize variations in the 5' flanking region of GH gene in different bovine species.

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#These authors contributed equally to this work.

MATERIALS AND METHODS

Applying simple random sampling in typical colony method in the central area of habitat, a total of 103 blood samples were obtained

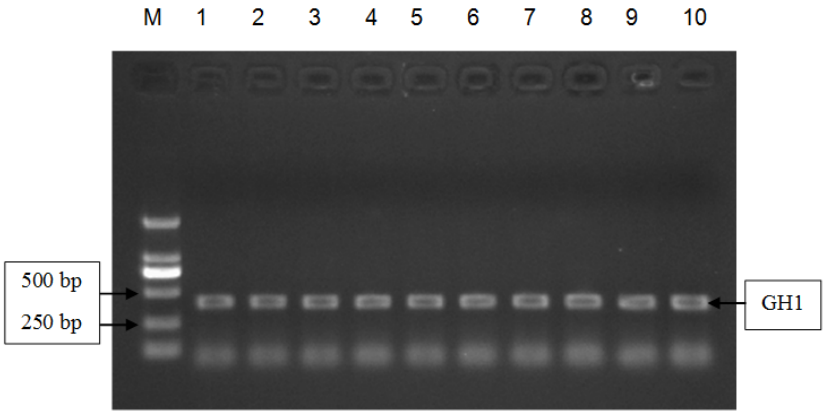

Figure 1. Electrophoretic patterns of the PCR products of the GH1 fragment (DL2000 DNA marker was used as contrast band).

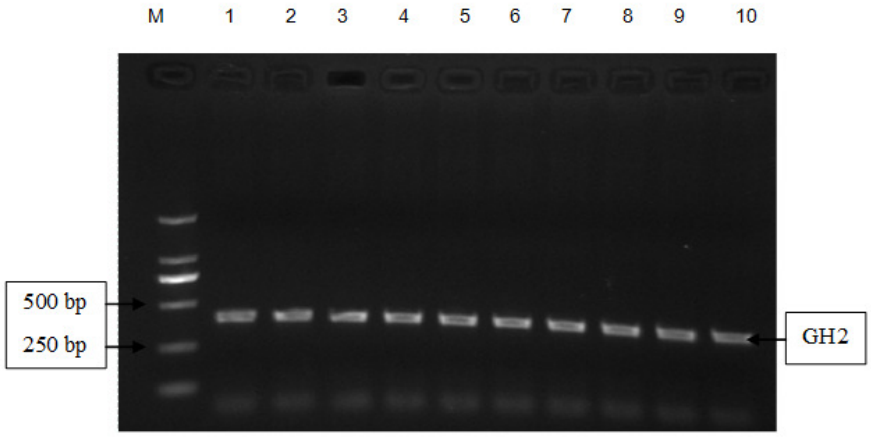

Figure 2. Electrophoretic patterns of the PCR products of the GH2 fragment (DL2000 DNA marker was used as contrast band).

from six bovine species, 18 samples of Leiqiong cattle (*Bos indicus*) from Guangzhou province, 19 samples each of Mangolian cattle (*Bos taurus*) and Bazhou yak (*Bos grunniens*) from Xinjiang autonomous region, 13 Gayal (*Bos frontalis*) from Yuunnam province, 18 Haizi buffalo (*Bubalus bubalis*) from Jiangsu province and 16 Nili-Ravi buffalo (*Bubalus bubalis*) from Guangxi province. These flocks were raised in the region of semi-agricultural and semi-pastoral or agricultural areas by natural grazing. The sampling was carried out in spring in the same year. Genomic DNA was isolated from the blood samples by a standard phenol/chloroform protocol.

Two pair of primers for PCR amplification and sequencing was designed according to published nucleotide sequence information of the bGH gene (Gordon et al., 1983). One amplified fragment was named as GH1 (the PCR primer pair were P1 5'-GGTGGGTTGCCTTTCTCTTCT-3' and P2 5'-TGTCATCA-TCCCGTCTCCACT-3'), including partial 5' flanking region. The other amplified fragment was named as GH2 (the PCR primer pair were P1 5'-TCTCAAGCTGAGACCCTGTGT-3' and P2 5'-GGCCAAATGTCTGGGTGTAGA-3'), including a region from 5' flanking region to part of the first intron.

PCR was performed using an initial denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 50s, annealing at 60°C for 50s and extension at 72°C for 60s, ending with a final extension at 72°C for 8 min, in a GeneAmp PCR system 9700 (Applied Biosysems, Foster, CA, USA).

Amplified DNA fragments were subjected to electrophoresis on a 1% agarose gel and target fragments were cut from gel for purify with spin columns (Watson Biotechnologies, Shanghai, China). Purified fragments were cloned into PMD 18-T Vector (Takara, Dalian, China) before sequencing. Sequencing was performed in both directions using an ABI PRISM 3730 BigDye Terminator Cycle Sequencing Kit (Applied Biosysems, Foster, CA, USA).

Sequences were manually trimmed to remove vector sequences and sequences were assembled with the DNASTAR software. Sequences were aligned by Clustalx1.83 package and the polymorphism results were exported using MEGA 4.0 (Tamura et al., 2007).

RESULTS AND DISCUSSION

Two kind of fragments GH1 (Figure 1) and GH2 (Figure 2) were amplified in all samples from six bovine species. In the GH1 fragment, three different types in length (465,

Table 1. Distribution of genotype frequencies of GH2 fragment of different bovine species.

Bovine species	GH1-A	GH1-B	GH1-C	GH1-D	GH1-E	GH1-F	GH1-G	GH1-H
Bos taurus	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bos indicus	0.77	0.06	0.17	0.00	0.00	0.00	0.00	0.00
Bos grunniens	0.95	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Bos frontalis	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00
Bubalus bubalis (Swamp buffalo)	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Bubalus bubalis (River buffalo)	0.00	0.00	0.00	0.00	0.00	0.00	0.87	0.13

Table 2. Distribution of genotype frequencies of GH2 fragment of different bovine species.

Bovine species	GH2-A	GH2-B	GH2-C	GH2-D	GH2-E	GH2-F
Bos taurus	1.00	0.00	0.00	0.00	0.00	0.00
Bos indicus	0.00	0.06	0.94	0.00	0.00	0.00
Bos grunniens	1.00	0.00	0.00	0.00	0.00	0.00
Bos frontalis	0.00	0.00	0.00	0.08	0.92	0.00
Bubalus bubalis (Swamp buffalo)	0.00	0.00	0.00	0.00	0.00	1.00
Bubalus bubalis (River buffalo)	0.00	0.00	0.00	0.00	0.00	1.00

467 and 473 bp) were identified according to sequencing results. The difference in length was due to a TG deletion sequence raised in Leiqiong cattle, while an ACTGCT insertion was raised in Haizi buffalo and Nili-Ravi buffalo. Twenty-six polymorphism sites were identified and denominated as GH1-A, GH1-B, GH1-C, GH1-D, GH1-E, GH1-F, GH1-G and GH1-H genotypes (Table 1). *Bos taurus*, *Bos indicus* and *Bos grunniens* shared genotype GH1-A and possessed the most frequency within population, while *Bos frontalis* and *Bubalus bubalis* possessed unique genotypes respectively.

In the GH2 fragment, two different types in length (450 and 453 bp) were identified according to sequencing results. The difference in length was due to an AGA deletion sequence raised in Leiqiong cattle, Haizi buffalo and Nili -Ravi buffalo. Only seven polymorphism sites were identified and denominated as GH2-A, GH2-B, GH2-C, GH2-D, GH2-E and GH2-F genotypes (Table 2). Except *B. taurus* and *B. grunniens* shared genotype GH2-A and possessed 100% frequency within population, *B. indicus*, *Bos frontalis* and *Bubalus bubalis* all possessed unique genotypes.

Through assembling fragment GH1 and GH2, we got the sequences of 5' flanking region of bGH gene, which cover almost the entire length. There was difference in length of 5' flanking region among different bovine species because of the insertion and deletion. Except the mutation of insertion and deletion, total thirty one variation sites were identified in this region. The predominant substitution model of nucleotide was transition and transition was higher than transversion with the ratio of 1.6.

Multiple alignment results are shown in Figure 3. Regarding the single nucleotide polymorphisms (SNPs), no variation was observed within Haizi buffalo. Compared with published bGH gene M57764, one, four, three, one and one new SNPs were identified within Mangolian cattle, Leiqiong cattle, Bazhou yak, Gayal and Nili-Ravi buffalo, respectively.

Analysis of sequences upstream of the ATG start site of the human and other animal GH genes has revealed various regulatory sequences (Wallis et al., 2001). Such regulatory elements of TATA box, CRE, NRE3, dPit1 and pPit1 were all identified in the six bovine species. The TATA box was named for its conserved DNA sequence, which is most commonly TATAAA, and many eukaryotic genes had a conserved TATA box. The TATA box element within bGH gene promoter was strongly conserved. A cyclic AMP response element (CRE) was found between the Pit-1 sites in the human GH gene promoter (Eberhardt et al., 1996) and appeared in the corresponding region in six bovine species. NRE3 probably represented a binding site for transcription factor YY1 (Park and Roe, 1996) and was conservative in all bovine species. Two putative binding sites for the Pit1 transcription factor (Theill and Karin, 1993) of other mammalian GH genes (Krawczak et al., 1999) were observed in the corresponding positions in bGH, the distalone overlapping the NRE3. It is notable that the distal and proximal sites in bovine species were very close to each other, but no changes in recognized regulatory elements in the promoter region, suggesting that regulation of bGH gene may be considerably conservative during the course of evolution. It was

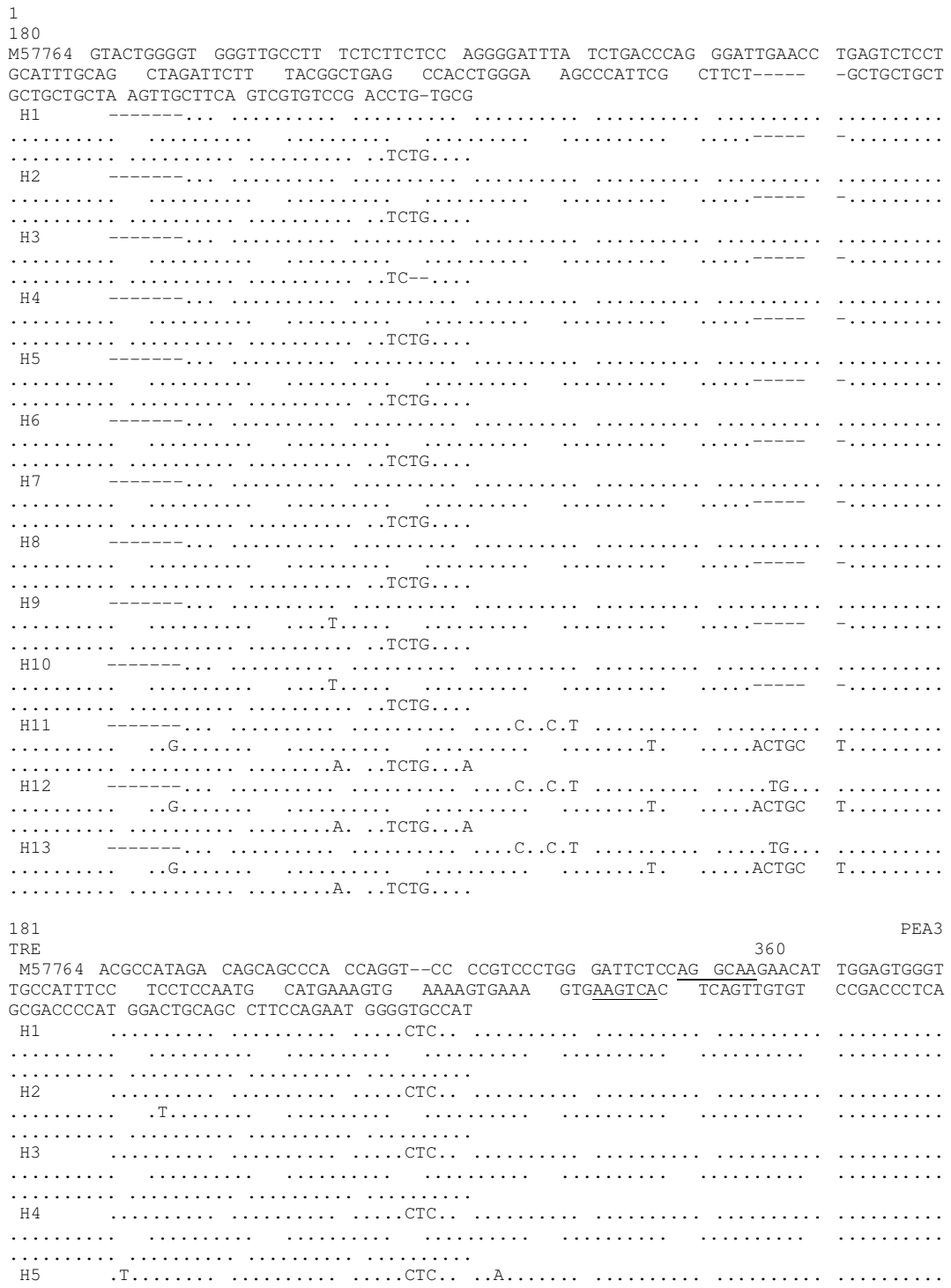

Figure 3. Comparison of the nucleotide sequences of bovine GH gene 5' flanking region (compared with GenBank accession No. M57764). Positions of regulatory elements are shown as discussed in the text. "." Represents identity to bovine GH sequence, "-" represents a deletion. H1 and H2, sequences from *Bos taurus*; H3 to H6, sequences from *Bos indicus*; H7 and H8, sequences from *Bos grunniens*; H9 and H10 represent sequences from *Bos frontalis*; H11, sequence from swamp buffalo (*Bubalus bubalis*); H12 to H13 represent sequences from river buffalo (*Bubalus bubalis*).

notable that the number of substitutions outside the defined elements also showed very strict conservatively within species and among species.

In addition, some SNPs were located near binding site

of transcription factor PEA3 (Roth et al., 1990) and transcription factor TRE (Theill and Karin., 1993). However, these transcription factor binding sites were scattered within a SINE/BovA2 repeatitive region. Previous studies (Ge et al., 2003; Ferraz et al., 2006) may explain the high variability in this region, and this region may not really be the functional promoter for the bGH gene. A palindrome was also identified between pPit1 and TATA box, which maybe a regulatory sequence.

Conclusion

Great differences appear in the distribution of genotypes of bGH gene 5' flanking region among Chinese bovine species. It seems related to species differences and living environment to a certain extent. It also may be very important to adapt the living conditions and play a vital role for growth and development. The 5' flanking region of bGH gene has a considerable number of mutations and although they are not located in known binding sites, they should be tested in transient expression assays using a gene reporter, in order to analyse their potential transcriptional activity. Given the mutations of the bGH gene is likely to influence growth and development, it may therefore be an indicator that could be used for the genetic improvement of bovine quantitative traits. Combining bGH gene polymorphism data with breeding value information could conceivably improve genetic selection in beef cattle production and increase the potential economic benefit to the beef cattle industry.

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