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Molecular Evolution of Coding and Non-coding Sequences of the Growth Hormone Receptor (GHR) Gene in the Family Bovidae*

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The GHR gene exon 1A and exon 4 with fragments of its flanking introns were sequenced in twelve Bovidae species and the obtained sequences were aligned and analysed by the ClustalW method. In coding exon 4 only three interspecies differences were found, one of which had an effect on the amino-acid sequence – leucine 152 proline. The average mutation frequency in non-coding exon 1A was 10.5 per 100 bp, and was 4.6-fold higher than that in coding exon 4 (2.3 per 100 bp). The mutation frequency in intron sequences was similar to that in non-coding exon 1A (8.9 vs 10.5/100 bp). For non-coding exon 1A, the mutation levels were lower within than between the subfamilies Bovinae and Caprinae. Exon 4 was 100% identical within the genera *Ovis*, *Capra*, *Bison*, and *Bos* and 97.7% identical for *Ovis moschatus*, *Ammotragus lervia* and Bovinae species. The identity level of non-coding exon 1A of the GHR gene was 93.8% between species belonging to Bovinae and Caprinae. The average mutation rate was 0.2222/100 bp/MY and 0.0513/100 bp/MY for the Bovidae GHR gene exons 1A and 4, respectively. Thus, the GHR gene is well conserved in the Bovidae family. Also, in this study some novel intraspecies polymorphisms were found for cattle and sheep.

Key words: GH receptor gene, Bovidae, polymorphism, sequence, alignments, evolution.

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The growth hormone receptor (GHR), a cell-surface mediator of actions of growth hormone, is a member of the cytokine/hematopoietin superfamily of receptors. In cattle, GHR is encoded by a single gene located on chromosome 20 (MOODY *et al.* 1995). The gene coding for bovine GHR consists of nine exons (numbered 2 to 10) in the translated part and a long 5'-noncoding region that includes nine untranslated exons – 1A through 1I (JIANG & LUCY 2001). Among them, only exons 1A, 1B, and 1C are well characterised; the existence of exons 1D to 1I is based on RACE (rapid amplification of cDNA end) analyses only. Exons from the untranslated region are spliced alternatively resulting in mRNAs differing in the 5'-untranslated region (5'-UTR). Exon 2 encodes a signal peptide, exons 3-7 – the extracellular GH-binding domain, exon 8 – the transmembrane domain, and exons 9-10 – an intracellular domain.

Several polymorphic sites in the 5'-noncoding region and in exon 10 of the bovine GHR gene have been identified (AGGREY *et al*. 1999; BLOTT *et al*. 2003; FALAKI *et al*. 1996; GE *et al*. 2003; MAJ & ZWIERZCHOWSKI 2002). One of the sequence variations, the T/A transversion in exon 8, results in an amino acid F279Y substitution in the transmembrane domain of the receptor. This mutation was shown to have a strong effect on the yield and composition of milk in Holstein-Friesian and Jersey cattle (BLOTT *et al*. 2003). To date, no polymorphisms have been reported in Bovidae species for exon 1A and exons 3 -7 coding for the extracellular domain of GHR.

The Bovidae family consists of 137 modern-day species distributed in 45 genera (GRUBB 1993). Within Bovidae, there are at least five subfamilies and as many as 14 tribes (ALLARD *et al.* 1992; NOWAK & PARADISO 1983; MEESTER & SETZER 1971; SIMPSON 1945; VAUGHAN 1978). Two widely recognized subfamilies are the Bovinae (e.g., cattle, bison, buffalos) and Caprinae (e.g., sheep, goats).

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The aim of the present work was to search for intra- and interspecies sequence variation in the Bovidae GHR gene, to detect mutations which occurred in the course of evolution in twelve species of the Bovidae family, and to compare mutation levels between coding exon 4 and non-coding exon 1A of the GHR gene.

Materials and Methods

DNA samples from five species belonging to the subfamily Bovinae*:* taurine cattle (*Bos taurus*), zebu (*Bos indicus*), European bison (*Bison bonasus*), bison (*Bison bison*), and water buffalo (*Bubalus bubalis*), and from seven species belonging to subfamily Caprinae: goat *(Capra hircus*), markhor (*Capra falconeri*), sheep (*Ovis aries*), mouflon (*Ovis musimon*), musk ox (*Ovibos moschatus)*, goral (*Naemorhedus goral*), and aoudad *(Ammotragus lervia)* were used for sequencing of a 466-bp fragment of the GHR gene, comprising the untranslated exon 1A, and a 444-bp fragment comprising 118 bp of intron 3, the entire exon 4, and 196-bp of intron 4. The sequence for *Bos taurus* exon 1A was taken from GenBank (accession no. U15731).

DNA was isolated from blood samples of goats, cattle, and European bison by the method of KANAI *et al*. (1994). DNA of other species was isolated from hair, using Genomic DNA Extraction Mini prep System (Viogene-Biotek Corporation, Taipei County, Taiwan).

Based on the ovine GHR gene sequence (O'Mahoney *et al*. 1994), and using the Primer3 software (www.genome.wi.mit.edu), primers were designed for PCR amplification of exon 1A: Forward: 5'-GTGATTGGGAGGGAGGAAGAGA-3'; Reverse: 5'-CAAGG-AGGGAGGGAGGAATAAAG-3'. For amplification of exon 4 with fragments of flanking introns primers GHREx4-F and GHREx4-R, published by BLOTT *et al*. (2003), were used.

Polymerase chain reactions were performed using a PCR-mix with: primers at 5.0 pmol/ml, 1 U Taq polymerase (Polgen, Łódź, Poland), 1 μl Taq polymerase buffer, four dNTPs, each at a final concentration of 0.2 mM, ca. 100 ng of genomic DNA, and H_2O to 10 μl. The reaction cycle consisted of an initial denaturation period at 95°C for 120 s, 36 cycles of 10 s denaturation at 95°C, 45 s annealing at 62°C, and 120 s elongation at 72°C, followed by 10 min final extension at 72°C. The PCR products were evaluated after electrophoresis in 2% agarose gel (Gibco) with ethidium bromide.

The PCR-amplified DNA fragments were eluted from agarose gels using GenElute™ Agarose Spin Columns (Sigma-Aldrich Corporation, St. Louis, Missouri, USA), purified with the GenElute PCR DNA Purification Kit, and sequenced at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland, on an ABI 377 sequencer (Applied Biosystems, Foster City, California, USA). Sequences were analysed using the Sequence Analyser 2.01 software.

Some of the nucleotide sequence variations were confirmed with restriction fragment length polymorphism (RFLP) methods. The amplified fragment containing ovine GHR gene exon 4 was digested with MspI. Restriction products were separated by electrophoresis in 2% agarose (Gibco-BRL, England) in 1 × TBE buffer (0.09 M Tris-boric acid, 0.002 M EDTA) with 0.5 μg/ml ethidium bromide (Et-Br), visualized under UV light, and scanned on a FX Phosphoimager apparatus (Bio-Rad, Hercules, Ca, USA).

Molecular phylogenies were derived using MEGA 2.1 software and maximum–parsimony (MP) analysis was performed using branch-and-branch search methods. Nonparametric bootstrap analyses were performed with 1000 replications. A total of 608 bp (including 210 bp of exon 1A, 130 bp of exon 4, 90 bp of intron 3, and 178 bp of intron 4) from GHR aligned as a tandem were used in the analysis.

For exon 1A numeration was started from the first nucleotide, for exon 4 and for introns the numeration followed that of BLOTT *et al*. (2003); in introns 3 and 4 positions were relative to the start site or to the end of exon 4, respectively.

The amino acid sequences were predicted on the basis of the obtained nucleotide sequences. A comparison of the nucleotide and amino acid sequences was performed with the Sequencher software (Gene Codes Corporation, Ann Arbor, Michigan, USA) and ClustalW (THOMPSON *et al*. 1994). Searching for homologous protein sequences was done using BLAST (http://www.ncbi.nlm.nih.gov/blast).

Results

GHR gene exon 1A and exon 4 with fragments of its flanking introns were sequenced in twelve Bovidae species. The sequences were deposited in the GenBank database (accession numbers: AY641539, AY671801, AY739705-AY739711, AY775292-AY775301).

Sequence alignment is shown in Fig. 1. In coding exon 4 only three interspecies differences were found: C/T transitions at positions 144, 152, and 205. Only one of these substitutions had an effect on the amino-acid sequence; the result of the C→T substitution at position 152 is a leucine→proline replacement at position 51 of the GHR protein (L51P). Thus, only three mutations occurred within

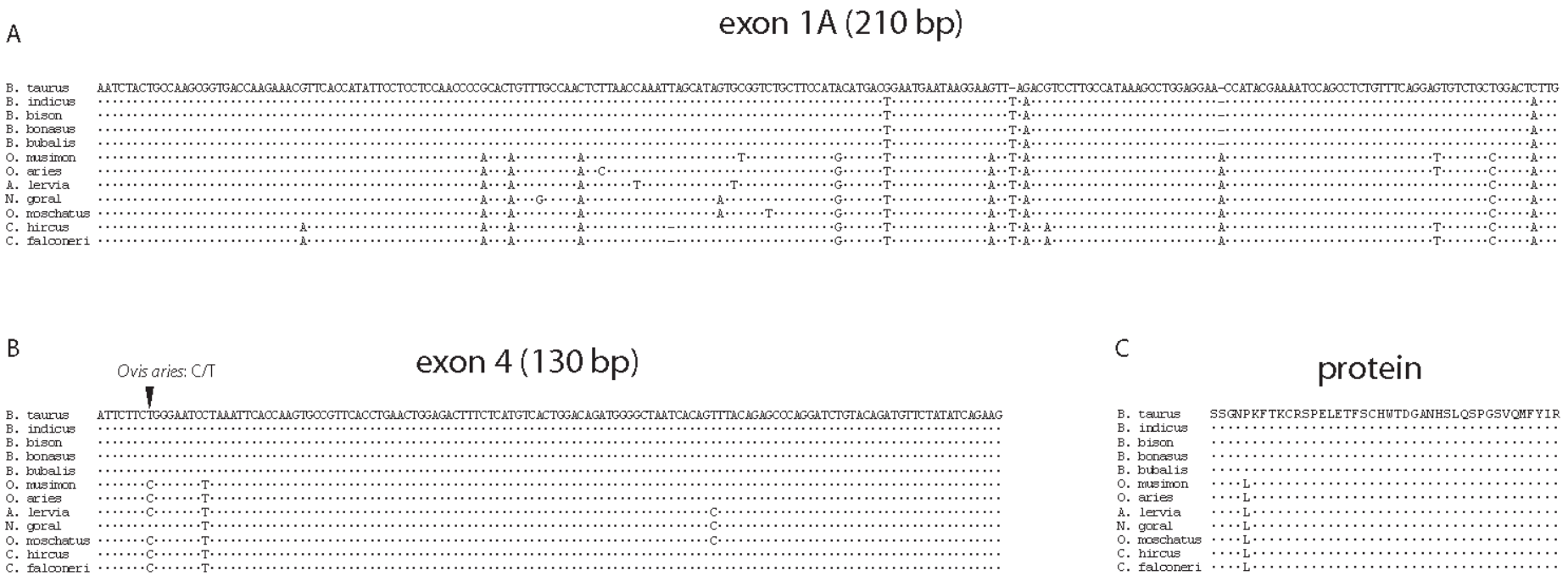

Fig. 1. Alignments of the relevant regions of the GHR gene of twelve Bovidae species. Nucleotide sequences of the bovine *GHR* gene exon 1A (A), exon 4 (B), and amino acid (a.a) sequence of the fragment of GHR extracellular domain encoded by exon 4 (C). Only differing nucleotides and a.a are shown by letters; identical nucleotides and a.a are shown by (•).

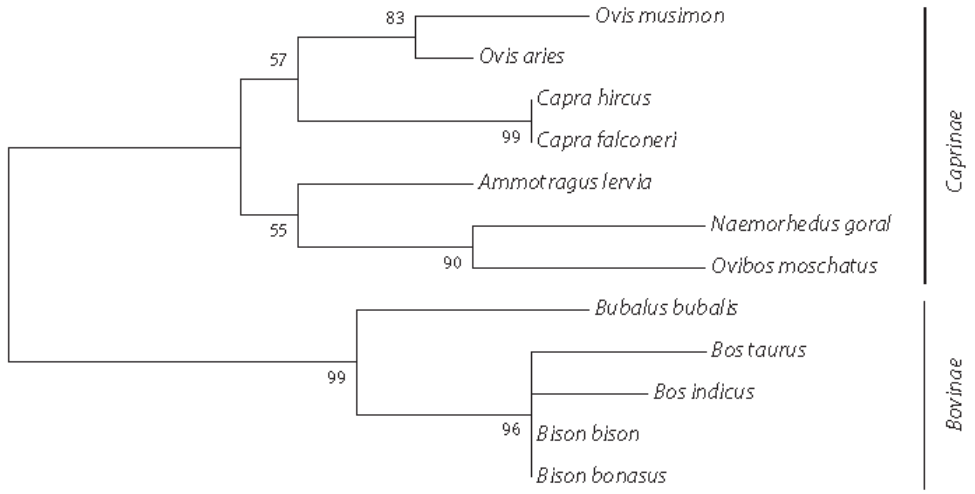

Fig. 2. Maximum–parsimony tree of GHR gene sequences of Bovidae. Bootstrap percentage values, indicating the degree of support for each cluster, are shown at the appropriate nodes. Branch lengths are proportional to the inferred percent change.

the 130-bp sequence (2.3/100 bp) of exon 4 coding for a part of the extracellular, ligand-binding domain of GHR (Fig. 1). The C/T substitution at position 152 was found only between subfamilies Bovinae and Caprinae, not within the subfamilies. Intraspecific polymorphism – a C/T transition at position 144 was found in the sheep GHR gene. This polymorphism was confirmed by digestion with *Msp*I. Two C/T heterozygotes and three C/C homozygotes were found after RFLP analysis of five sheep; no T/T homozygote was found. This polymorphism was found not only in sheep, but also was observed between species. Cytosine was typical for most Caprinae species (except for *Naemorhedus goral*), while T was found in all Bovinae species under study.

The nucleotide sequence conservation of exon 4 varied from 100% (among the genera *Ovis*, *Capra*, *Bison*, and *Bos)* to 97.7% (between *Ovibos moschatus, Ammotragus lervia* and Bovinae species).

In 210 bp of non-coding exon 1A, twenty-two interspecies differences were found (10.5/100 bp). There were nineteen nucleotide substitutions and three single-nucleotide deletions at positions +83, +132 and +162 (Fig. 1). Seven substitutions were found between, but not within, subfamilies. The T deletion at position +83 was specific for goat and markhor, while deletion of A at position +162 was found in the Bovinae subfamily only. The degree of identity of this fragment of the *GHR* gene was from 100% (among European bison, bison, zebu and water buffalo, and between goat and markhor) to 93.8% (between species belonging to the subfamilies Bovinae and Caprinae).

Nucleotide sequences of 90 bp of intron 3 and 178 bp of intron 4 were aligned as a tandem. Twenty-four interspecies differences were found (8.9/100 bp). There were twenty-one nucleotide substitutions and three di- and trinucleotide insertions/deletions at positions 137-13, 266+105 and 266+114, respectively. Two nucleotide substitutions were found between, but not within the subfamilies. A TG insertion at position 266+105 and a AGA insertion at position 266+114 are specific for goat and markhor; a GTCTT insertion at position 137-13 was found only in the genera *Bos* and *Bison*. Moreover, an intraspecies polymorphism was found in the bovine (*Bos taurus*) *GHR* gene intron 4 - a G/C transversion at position 266+29 and a G/A transition at position 266+72.

The degree of identity of analysed fragments of introns 3 and 4 was from 100% (within the genera *Capra* and *Bison)* to 94.7% (between *Capra hircus* and *Bos indicus)*.

A maximum parsimony phylogeny of the tandem alignment of exon 4, exon 1A, and fragments of introns 3 and 4 of the GHR gene in twelve representatives of the Bovidae family is shown in Fig. 2.

Discussion

In this study, sequences of coding and non-coding *GHR* gene fragments of twelve Bovidae species belonging to Bovinae and the Caprinae subfamilies were compared in order to identify mutations that appeared during the evolution of the family Bovidae. The average mutation frequency in non-coding exon 1A was 10.5 per 100 bp, and was 4.6-fold higher than that in coding exon 4 (2.3 per 100 bp). The mutation frequency in intron sequences was similar to that in non-coding exon 1 (8.9 vs 10.5/100 bp). For non-coding exon

1A the mutation levels were lower within than between the subfamilies Bovinae and Caprinae.

It was found (BASS *et al*. 1991; CLACKSON & WELLS 1995; DE VOS *et al*. 1992; WELLS 1996) that in the human GHR amino acids at positions 60, 61, 62 and 88, encoded by exon 4 are critical for the GH - GHR interaction. In the present work, no mutations were found in these positions in the *GHR* gene in the Bovidae species studied.

The results indicate that the leucine at position 51 of GHR is unique to Caprinae; all other vertebrate species available from the GenBank database, including fishes, contain proline in this position. No differences were found within the studied fragments between *C. hircus* and *C. falconeri*, considered a wild ancestor of the domestic goat.

The GHR gene has frequently been used to construct phylogenetic relationships among mammal taxons, especially rodents (ADKINS *et al*. 2001; ADKINS *et al.* 2003; ROWE & HONEYCUTT 2002). These authors demonstrated the potential for resolving relationships among higher taxonomic categories of mammals using type I genetic markers (coding sequences) and gave strong support to relationships among rodents. In this study sequences derived from the *GHR* gene were used to construct a phylogenetic tree for twelve species of two subfamilies of Bovidae. The present results obtained for nuclear genomic sequences agree with those from earlier studies using the mitochondrial cytochrome *b* gene (HASSANIN & DOUZERY 1999, HASSANIN *et al*. 1998).

Previously, polymorphisms were found in the bovine *GHR* gene exons 8 and 10, introns 2, 8 and 9 (BLOTT *et al.* 2003), and in the promoter P1 region (AGGREY *et al.* 1999; GE *et al*. 2003; MAJ & ZWIERZCHOWSKI 2002). No polymorphism has been reported to date for other Bovidae species. In this work two novel polymorphisms were found in the bovine *GHR* gene intron 4 and one polymorphism, the C/T transition in exon 4 of the ovine gene. The latter is the first SNP ever found in a Bovidae species other than *Bos taurus*. New polymorphisms are valuable tools in the search for genetic markers of animal productivity.

Most of the polymorphisms found in the present work are co-dominant, bi-allelic, single nucleotide polymorphisms (SNPs). Although less polymorphic, and thus less informative than type II markers (microsatellites), SNPs have gained popularity in genetic studies due to their high accuracy and reproducibility (VIGNAL *et al*. 2002).

The Bovidae subfamilies Bovinae and Caprinae diverged some 12-15 million years (MY) ago (HASSANIN & DOUZERY 1999). Therefore, the average mutation rate is 0.2222/100 bp/MY and 0.0513/100 bp/my for the Bovidae *GHR* gene exons 1A and 4, respectively. The assumed average gene mutation rate is 0.537 mutation/100 bp/MY for nucleotide changes at silent codon or intron positions (MIYATA *et al*. 1982). Thus, the *GHR* gene is well conserved in the Bovidae family, in particular its coding sequences.

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