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Short Communication

Identification of a DNA methylation point in the promoter region of the bovine *CYP21* gene

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ABSTRACT. The *CYP21* (steroid 21-hydroxylase) gene is involved in the synthesis of steroid hormones. Bov-A2 is a retroposon that is common in ruminant genomes. The promoter region of bovine *CYP21* contains a short interspersed nucleotide element of Bov-A2, which overlaps a putative Sp1 binding site. We looked for RFLP/*Hpa*II polymorphism in the Bov-A2 element in bovine Zebu breeds by PCR-RFLP, and examined whether polymorphism in this element is associated with methylation. Among DNA samples from 135 Brazilian Zebu breed cattle, we identified an RFLP/*Hpa*II polymorphism (T/C), which, based on a restriction methylation-sensitive assay employing *Hpa*II and isoschizomer *Msp*I enzymes (methylation-sensitive and -non-sensitive enzymes, respectively), appears to be a DNA methylation point. This

is the first report of this polymorphism and on DNA methylation in the bovine *CYP21* promoter region in Brazilian Zebu cattle.

Key words: *CYP21* gene; Bov-A2; SINE; Cattle; Epigenetics; Methylation

INTRODUCTION

The most common enzymatic defect of steroid synthesis is adrenal steroid 21-hydroxylase deficiency. The human *CYP21A2* gene is well known in terms of location, duplication in tandem with the fourth component (C4) isotypes, structure, and the sequencing of the gene and its pseudogene and their arrangement on the chromosome (New, 2006).

Several studies have been directed towards genetic variability in the regulatory region, where the presumed effect would influence the regulation of gene transcription (Samarin et al., 2009). The composition of the regulatory elements in vertebrates could be complex including modules of transcription factor binding sites, intergenic ncRNAs and ancient fragments derived from retrotransposition events (Nijman et al., 2002).

Variability in the regulatory sites of genes could affect the binding of regulatory proteins or erase methylation sites, as in the case of deamination in methylated CpG motifs (Gartler and Riggs, 1983). Retroelements are frequently associated with regulatory regions and are highly methylated (Kubis et al., 2003). This heavy methylation could be a source of genetic mutation by deamination at these elements giving rise to new alleles of these sequences (Wang and Leung, 2008).

According to Lenstra et al. (1993) there are three short interspersed nucleotide element (SINE) families in the Ruminantia: Bov-A2, Bov-tA, and Bov-B, which are estimated to account for 1.8, 1.6, and 0.5% of the bovine genome, respectively. The Bov-A2 element is composed of two monomers, called Bov-A units (a segment of 115 bp), joined by a linker sequence of short repeats (CACTTT)n, with approximately 220,000 copies in the entire genome (Onami et al., 2007). Damiani et al. (2000a) showed that the mutation frequency (substitutions and deletions/insertions per nucleotide site per year) of the Bov-A2 sequences is generally higher than those of other genomic noncoding sequences. This high amount of genetic variation in the Bov-A2 elements could be responsible for several processes such as recombination and gene conversion. The same authors suggested that a considerable number of point mutations, insertions, and deletions are present in the whole Bov-A2 sequence and, therefore, this retroelement may be an important source of single nucleotide polymorphisms (SNP) for the analysis of the Bovidae genomes. The effects of these chromosomal "parasites" on gene expression can provoke epigenetic and consequently phenotypic variability (Druker and Whitelaw, 2004).

SINEs contain internal regulatory sequences, which are presumed to influence the transcription of neighboring genes (Zwolińska, 2006). Transposable elements close to regulatory sequences of some genes in mice have been reported as influencing the host gene. There are no studies demonstrating that this specific Bov-A2 element can influence the expression of bovine *CYP21*. In mammals, retroelements account for 30-40% of the genome, and have two open reading frames and 5' and 3' untranslated regions allowing them to amplify themselves in the mammalian genome via reverse transcriptase and then modify the biology of cells by reinsertion and possibly by modulation of RNA biology (Ramos, 2009). Within the

last decade, researchers have moved away from the "selfish DNA" hypothesis (non-functional genomic residents) and have understood that mobile elements are important regions within genomes (Hizer et al., 2008). Many elements have been discovered in heterochromatin and other heavily methylated regions, presumably as a genomic response to suppress their activities as inspectional mutagens and/or as sites of recombination and rearrangement, considered to be deleterious to the host (Morse et al., 1988). Other beneficial roles of these elements have also been shown, such as DNA repair mechanisms, gene duplication, X-chromosome inactivation, telomere maintenance, and modulators of gene expression (Hizer et al., 2008).

The polymorphism in the promoter region of the bovine *CYP21* gene was first identified by Damiani et al. (2000b) through the polymerase chain reaction (PCR) amplification of a fragment containing the Bov-A2 element followed by direct sequencing of its product.

The aim of this study was to identify the occurrence of polymorphism RFLP/*Hpa*II (T/C change) of SINE elements (Bov-A2) located in the promoter region of the *CYP21* gene in Zebu breeds: Gir, Guzerat and Nellore. These breeds have considerable economic importance and can be used as animal models for human diseases and development. This study also demonstrated a possible methylation point in this SNP and the possibility of this polymorphic site being a target for epigenetic marks.

MATERIAL AND METHODS

Animals and DNA isolation

DNA was isolated from peripheral blood samples of 135 animals of *Bos taurus* and *Bos indicus* Gir (66), Guzerat (43) and Nellore (26). A total of 39 Gir animals belonged to the APTA (Agência Paulista de Tecnologia dos Agronegócios - Nordeste Paulista) and 27 belonged to a private farm. All Guzerat and Nellore animals belonged to farms participating in the Breeding Program for Nellore and Guzerat Cattle (PMGRN and PMGRG). Approximately 10 mL peripheral blood was drawn from each animal, by an authorized veterinarian, into tubes containing EDTA. DNA extraction was carried out following the modified protocols of Olerup and Zetterquist (1992).

Detection of polymorphism and genotyping

A 351-bp fragment comprising a portion of the promoter region of the bovine *CYP21* gene (Figure 1) was amplified by PCR. The primer sequences were obtained from the promoter region of the *CYP21* gene (GenBank accession number M11267). Sequences of the forward and reverse primers were 5' CCCACCGAGTCCTGCCAC 3' and 5' GAGGGGGCAGTTGAAGGAC 3', respectively. PCR was carried out in a final volume of 25 µL [50 mM $MgCl_2$, 1.5 µL 10X PCR buffer, 5 pmol of each primer, 0.25 mM dNTPs and 0.2 U Taq DNA polymerase (Invitrogen, Life technologies, Paisley, UK)]; 50 ng genomic DNA was used per reaction. The cycle conditions were 5 min at 94°C, followed by 40 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C), and a final extension step for 10 min at 72°C. The preceding thermal procedure was performed in a T Gradient thermocycler (Whatman Biometra, Gottingen, Germany). The PCR products were separated on 2% agarose gels and visualized using ethidium bromide and a gel documentation system. The PCR

fragments were digested in a final volume of 10 µL, containing 3 µL PCR product, 4 U restriction endonuclease *Hpa*II (New England Biolabs, Beverly, MA, USA) supplemented with the recommended 1X digestion buffer (NEBuffer 1.10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol). Digestion was performed at 37°C overnight, followed by heat inactivation at 65°C for 20 min. The products were separated and visualized by electrophoresis on a 10% acrylamide gel stained with 0.2% silver nitrate.

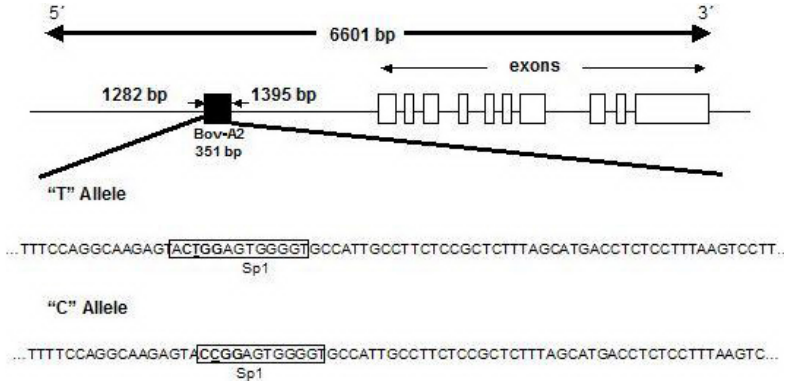

Figure 1. Schematic representation of the bovine *CYP21* gene sequence (GenBank accession number M11267) [Chung et al. (1986), modified]. The black box represents a 351-bp fragment localized in the promoter region of the bovine *CYP21* gene, and the open boxes show exons 1 to 10. The sequences show the SNP underlined (with RFLP/*Hpa*II polymorphism), overlapping a putative Sp1 transcription factor binding site (open box in sequences) (www.generegulation.com/pub/programs/alibaba2; http://tfbind.hgc.jp/).

In silico analysis of RFLP/*Hpa*II polymorphism

The sequences were analyzed using bioinformatics tools including search for identification of "Sp1" sites (Searching Transcription Factor Binding Sites). The search included identification of "Sp1" sites overlapping the target sequence (http://www.gene-regulation.com/pub/programs.html?PHPSESSID=4ed4f30e499140b61b02284a945a89db#alibaba2; http://tfbind.ims.u-tokyo.ac.jp).

Analysis of DNA methylation in the C allele

Genomic DNA samples (100 ng per reaction) of heterozygous Nellore, Gir and Guzerat animals were digested with the *Hpa*II enzyme (a methylation-sensitive endonuclease) and a separate sample was digested with the *Hpa*II isoschizomer, *Msp*I (which is a methylation-non-sensitive endonuclease), prior to PCR. The undigested control and the digested *Hpa*II and *Msp*I samples were amplified with the primers for the bovine RFLP/*Hpa*II polymorphism. The PCR products were submitted to a second round of digestion with the *Hpa*II enzyme to determine the methylation status of the "C allele" in this specific Bov-A2 element.

RESULTS

An RFLP/*Hpa*II polymorphism (T/C change) of the Bov-A2 element in the promoter

region of the *CYP21* gene was identified in the cattle (Figure 1), with allelic frequencies of 19, 20 and 44% (C) and 81, 80 and 56% (T), respectively, for Gir, Guzerat and Nellore (Table 1). High genotypic frequencies of heterozygotes (CT) and homozygotes (TT) were observed (Figure 2). Figure 1 shows the SNP, with the RFLP/*Hpa*II polymorphism underlined, overlapping a putative Sp1 transcription factor binding site.

Table 1. Allelic frequencies for RFLP/*Hpa*II polymorphism in Gir, Guzerat and Nellore animals.

Breed	N	Allelic frequency	
		C	T
Gir	66	0.1894	0.8106
Guzerat	43	0.1970	0.8030
Nellore	26	0.4423	0.5577
Total	135	0.2741	0.7260

N = total number of animals.

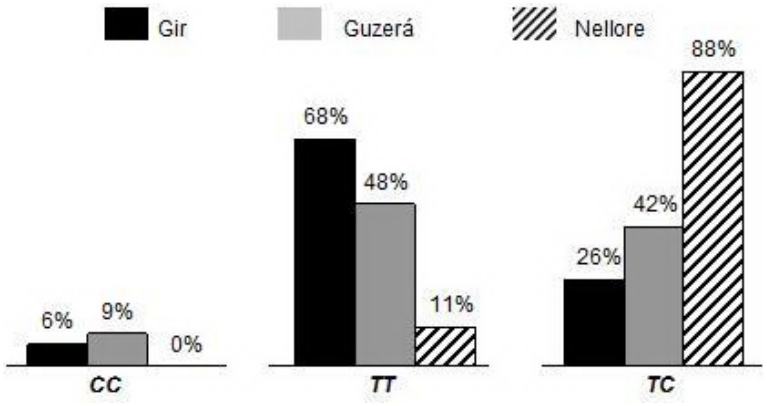

Figure 2. Genotypic frequencies observed in the digestion of PCR products with the enzyme *Hpa*II of DNA samples from Gir, Guzerat and Nellore breeds.

The results of the allele-specific methylation assay demonstrated that the cytosine residue in the CpG motif, when it occurs in the C allele, is methylated in all heterozygous Nellore, Gir and Guzerat animals (Figure 3).

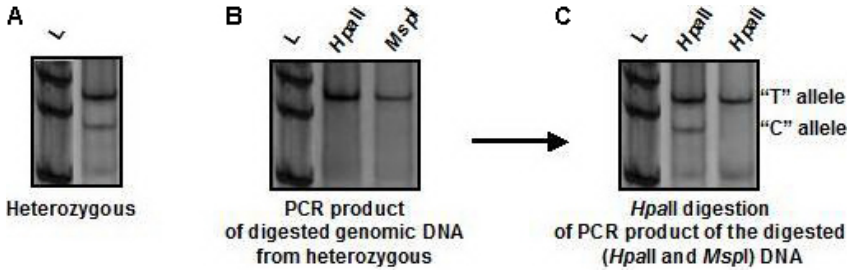

Figure 3. Allele-specific methylation assay. **A.** Genomic DNA of heterozygous animals digested with *Hpa*II and *Msp*I enzymes, prior to PCR. **B.** PCR of samples digested. **C.** The PCR products submitted to a second round of digestion with the *Hpa*II enzyme to determine the methylation status of the "C allele". L = 100-bp DNA marker.

DISCUSSION

The genotypic frequencies showed a high percentage of heterozygote (CT) and homozygote (TT) animals in all breeds studied (Figure 2). It is not yet clear if this Bov-A2 element and its polymorphism may have any effect on the expression of the *CYP21* gene. The allele-specific methylation assay (Figure 3) demonstrated that the cytosine residue in the CpG motif, when it occurs in the C allele, is methylated in all heterozygous animals. The T>C mutation could also create a CpG motif, which could be a possible DNA methylation site. Transitional CpG methylation between unmethylated promoters and nearby methylated retroelements plays a role in the establishment of tissue-specific transcription. A series of evidence show that retroelement methylation is prevented on the unmethylated CpG islands and promotes the methylation of the non-island-CpG sites (Jung et al., 2008). Aberrant DNA methylation patterns in the retroelement sequences are thought to permit the reactivation of transpositional events and allow regulatory elements into the "parasites" that act on the transcriptional control of the neighboring genes (Martin, 2006). Some classic examples of aberrant gene regulation influenced by gene-associated retrotransposons are reported in the mouse *agouti viable yellow* (A^{vy}) and *axin fused* ($Axin^{Fu}$) alleles (Dolinoy et al., 2007). It is unknown if a similar effect occurs in cattle, but the erasing of the CpG motif on a putative Sp1 binding site in a promoter region could provide a candidate model for this event. The SNP (RFLP/*Hpa*II polymorphism) is overlapped by a putative Sp1 transcription factor binding site (Figure 1). The polymorphic site to Sp1 protein (specific protein Sp1) provides protection against methylation and is a transcription factor with the function of maintenance of regulatory sequences (Frank et al., 1991). Several maintenance genes display a GC-rich space of 20-50 nucleotides recognized by a family of Sp1 transcription factors. Boumber et al. (2008) observed that the Sp1 site is considered to be a regulator of DNA elements, protecting CpG islands against methylation during embryogenesis. The genetic and epigenetic features of this SNP will contribute to a possible functional effect on the regulation of the bovine *CYP21* gene.

The present study determined the frequencies (homozygote and heterozygote) of the RFLP/*Hpa*II polymorphism in Brazilian Zebu breeds (*Bos taurus* and *Bos indicus*) and is the first association of the genetic variability of this locus as a possible source of epigenetic variability at the same genomic position. The three genotypes CC, CT and TT for this locus should influence bovine *CYP21* expression, possibly by genetically mediating epigenetic variability.

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