SHORT COMMUNICATION

Characterization and polymorphism screening of IGF-I and prolactin genes in Nelore heifers

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ABSTRACT

Insulin growth factor I (IGF-I) and prolactin (PRL) are peptide hormones that exert complementary effects on reproductive traits by acting on folliculogenesis. In view of the lack of information about the IGF-I and PRL genes in *Bos indicus*, the objective of this study was to partially characterize the promoter regions of these genes and to screen animals of different ages at first pregnancy for the presence of polymorphisms in these regions. In addition, we determined whether polymorphisms influence the regulation of the two hormone genes, evaluating their association with sexual precocity.

The animals were divided into three groups according to age at first pregnancy: 1) 100 heifers considered to be sexually precocious that became pregnant at 15-16 months of age, 2) 100 heifers that became pregnant during the normal breeding season at 24 months of age, and 3) 100 heifers that did not become pregnant until 24 months of age. For the IGF-I gene, PCR-RFLP-*SnaB*I analysis showed the presence of genotypes AB and BB at frequencies of 0.02 and 0.98, respectively. Sequencing of the IGF-I gene fragment revealed a single nitrogen base change from cytosine to thymine, corresponding to the restriction site of *SnaB*I. The polymorphisms identified in the 5'-flanking region of the IGF-I gene may serve as a ba-

sis for future studies of molecular markers in cattle. For the PRL gene, PCR-RFLP-*Hae*III analysis showed the presence of only one migration pattern, a finding characterizing the region studied as monomorphic. The study of other regions in the IGF-I and PRL genes might provide molecular data that can be used in the future for the selection of sexually precocious animals.

Key words: Beef cattle, Molecular marker, RFLP, Sexual precocity.

RIASSUNTO

CARATTERIZZAZIONE E RICERCA DI POLIMORFISMI DEI GENI IGF-I E PROLATTINA IN GIOVENCHE DI RAZZA NELORE

Gli ormoni IGF-I e Prolattina hanno un effetto complementare nella riproduzione, agendo sul processo di follicologenesi. Considerando la limitatezza di informazioni sui geni IGF-1 e Prolattina nel Bos indicus, questo studio è stato condotto con l'intento di caratterizzare la regione promotrice di questi geni e investigare l'esistenza di polimorfismi in queste regioni, in gruppi di animali con diverse età alla prima gravidanza. Inoltre è stato valutato se i polimorfismi possono influenzare la regolazione di questi geni, valutando la loro associazione con la precocità sessuale.

Gli animali sono stati divisi in tre gruppi in base all'età alla prima gravidanza: 1)100 manze considerate precoci ingravidate a 15-16 mesi d'età, 2) 100 manze che hanno concepito normalmente nella stagione di monta a 24 mesi d'età e 3) 100 manze che fino a 24 mesi d'età non avevano ancora concepito. Per il gene IGF-I, l'analisi con PCR-RFLP-SnaBI ha permesso di osservare la presenza dei genotipi AB e BB con rispettiva frequenza di 0,02 e 0,98. Il sequenziamento del frammento del gene IGF-I ha mostrato il cambiamento di una singola base azotata da citosina a timina, corrispondente al sito di restrizione dell'enzima SnaBI. I polimorfismi identificati nella regione fiancheggiante 5' del gene IGF-I possono servire come base per altri studi di marcatori molecolari nei bovini. Per il gene della Prolactina l'analisi PCR-RFLP-HaeIII ha mostrato la presenza di un solo tipo di pattern di migrazione, caratterizzando la regione studiata come monomorfica.

Lo studio di altre regioni nei geni IGF-I e Prolattina potrebbe produrre dati molecolari da utilizzarsi in futuro per la selezione di animali sessualmente precoci.

Parole chiave: *Bovini da carne, Marcatori molecolari, RFLP, Precocità sessuale.*

Introduction

Reproductive performance of animals is one of the main traits that affect the economic efficiency of animal production systems. Beef cattle herds consisting of animals that present a high frequency of sexual precocity and fertility are characterized by a younger mean age at first calving, increased availability of animals for both sale and selection, higher meat production and, consequently, greater profitability. Thus, sexually precocious animals are desirable and sexual precocity-related traits should be included in selection programs. The lack of reproductive traits in the selection index applied may substantially decrease the economic efficiency of selection (Newman *et al*., 1992). However, the inclusion of sexual precocity in animal breeding programs is difficult since this trait it is not easily measured. In addition, reproductive traits usually present low heritability estimates and do not respond rapidly to selection (Mercadante *et al*., 2000). In the case of Brazilian *Bos indicus* cattle, selection of sexually precocious animals represents a great challenge for breeders since these animals enter the reproductive phase later than animals of European breeds (Cundiff, 2004).

Molecular studies for the identification of polymorphisms in genes related to the physiological regulation of reproductive processes may contribute to the selection

of traits such as sexual precocity. The use of polymorphisms in candidate genes for marker-assisted selection may increase the genetic gain achieved by selection as a result of more accurate predicted breeding values (Parmentier *et al*., 1999). Thus, the development of genome maps by molecular analysis raises the possibility of the identification of genes that control reproductive efficiency, thus permitting the use of marker-assisted selection as an auxiliary method in the selection of more sexually precocious *Bos indicus* animals.

Possible candidate genes related to sexual precocity include the insulin-like growth factor I (IGF-I) and prolactin (PRL) genes because both hormones exert a complementary effect on reproduction by acting on the process of folliculogenesis (Nolin *et al*., 1980; Savion *et al*., 1981; Spicer *et al*., 1993; Schams *et al*., 1999; Chandrashekar and Bartke, 2003; Phelps *et al*., 2003).

In view of the lack of information about the IGF-I and PRL genes in *Bos indicus*, the objective of this study was to partially characterize the promoter regions of these genes and to screen animals of different ages at first pregnancy for the presence of polymorphisms in these regions. In addition, we determined whether polymorphisms influence the regulation of the two hormone genes, evaluating their association with sexual precocity.

Material and methods

Hair samples from 300 Nelore females were collected. The animals were divided into three groups according to the confirmation of pregnancy of the heifers: 1) 100 heifers considered to be precocious, which became pregnant at 15-16 months of age, 2) 100 heifers that became pregnant during the normal breeding season at 24 months of age, and 3) 100 heifers that did not become pregnant. Genomic DNA was extracted from hair follicles obtained from the tail brush of the heifers (about 40 follicles/animal) as previously described by Dias-Cano & Brady (1997), but using 500μL TE-Tween (50mM Tris, 1mM EDTA, 0.5% Tween 20) instead of SDS.

The promoter regions of the IGF-I and PRL genes were isolated using the polymerase chain reaction (PCR). The following primers were used for amplification of the IGF-I gene: 5'–ATT-ACA-AAG-CTG-CCT-GCC-CC–3' (forward) and 5'–ACC-TTA-CCC-GTA-TGA-AAG-GAA-TAT-ACG-T–3' (reverse) (Invitrogen, Carlsbad, CA, USA) as described by Ge *et al*. (2001). For the PRL gene, 5'–ATG-ATT-TTT-GGT-GGC-CCT-AG–3' (forward) and 5'–TGA-GCA-GGA-GAT-GGA-GAG–3' (reverse) (Invitrogen) primers were designed from the promoter region sequence available in GenBank (accession No. AB098480). PCR was carried out in a final volume of 25μL containing 100ng DNA, 0.5μM of each primer, PCR buffer (10mM Tris-HCl, pH 9.0, and 50mM KCl), 1.5mM $MgCl_2$, 100μM dNTPs, and 0.5U Taq polymerase (Invitrogen). The PCR conditions were: an initial denaturation step at 95°C for 5min, followed by 35 cycles at 94°C for 1 min, 54°C (PRL primers) or 56°C (IGF-I primers) for 1 min, and 72°C for 1 min.

The PCR fragments of the IGF-I and PRL genes were analyzed by RFLP, with digestion of the fragments with *Sna*BI (Promega, Madison, WI, USA) and *Hae*III (Invitrogen) restriction endonucleases, respectively, at 37°C for approximately 1h. The cleaved fragments were separated by electrophoresis on 3.5% agarose gel in 1X TBE buffer (89mM Tris-HCl, 2.5mM EDTA and 89mM boric acid, pH 8.3) containing 0.05μg/mL ethidium bromide at 75V for approximately 2h. The bands were visualized under ultraviolet light in a Gel-Doc system (Bio-Rad, Hercules, CA, USA).

Gene and genotype frequencies were estimated by simple counting of the alleles based on the electrophoresis results using the equations described by Falconer and Mackay (1996). Three random samples of the PRL gene region and five samples of the IGF-1 gene, with two samples showing the polymorphic site obtained by PCR-RFLP, were sequenced. The PCR products of the PRL and IGF-I genes were ligated into plasmids using the pGEM®-T-Easy Vector Systems kit (Promega). After ligation, the recombinant vectors were inserted by transformation reactions into *E. coli* DH10B competent cells. After this step, colonies of cells were cultured and plasmid DNA containing the PCR fragments was identified by white color and extracted as described by Sambrook and Russel (2001).

Cloned fragments of the two genes were sequenced from the 5' to 3' end using the BigDye Terminator Cycle Sequence Ready ABI Prism kit, version 3, in an automatic ABI PRISM 3700 sequencer and analyzed using the CodonCode Aligner and CLC Free WorkBench3 softwares.

Results and discussion

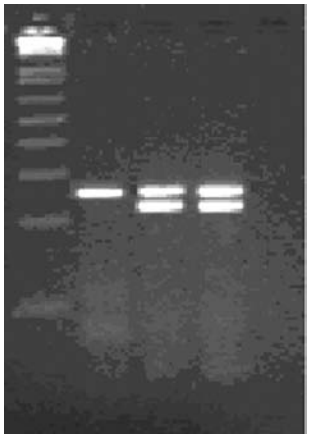
PCR-RFLP digestion of the 249-bp fragment obtained from the 5'-flanking region of the IGF-I gene with *SnaB*I resulted in two migration patterns. According to Ge *et al*. (2001), digested samples that result in three fragments (249, 226 and 23 bp) correspond to genotype AB and undigested samples (249pb) are genotype BB (Figure 1). The frequency of genotypes AB and BB detected by RFLP was 0.02 and 0.98, respectively. For the PRL promoter region, digestion of the 548-bp fragment with *Hae*III resulted in bands of 291 bp and 257bp. Heifers of all experimental groups presented the same electrophoretic migration pattern, called genotype BB.

RFLP-*SnaB*I analysis of the IGF-I gene revealed the BB genotype in all females of the early pregnant and nonpregnant groups. Only two females of the group that became pregnant at 24 months of age presented the AB genotype, corresponding to a genotype frequency of 0.02. Ge *et al*. (2001), studying the same IGF-I gene polymorphism in 760 Angus calves, detected three different genotypes (AA: 0.43; AB: 0.42; BB: 0.15) and observed a significant association between this RFLP and growth traits. The present results are in contrast to those reported in the study of Ge *et a*l. (2001) in which the B allele showed a lower frequency in a *Bos taurus* population. This fact may indicate that this allele (B) is characteristic of *Bos indicus* animals. In addition, the presence of the polymorphic allele (A) at low frequency in the *Bos indicus* animals analyzed suggests the participation of the *Bos taurus* genome in the formation of the Nelore breed. Meirelles *et al*. (1999) estimated a major participation of matrilineages of taurus origin in purebred *Bos indicus* originating from the American continent based on the analysis of microsatellite markers in the mitochondrial DNA of these animals.

According to Casas *et al*. (2005), *Bos indicus*-specific markers should be developed since the differences in allele frequencies observed between *Bos taurus* and *Bos indicus* may influence markers initially validated for *Bos taurus*. On the other hand, these authors reported the existence of possible similar sources of variation in *Bos taurus* and *Bos indicus*. Investigation of these sources might be used to develop markers that could be applied to both species.

The predominance of the BB genotype observed when analyzing the 5'-flanking region of the IGF-I gene by RFLP may also be related to the fact that this population has been selected for growth traits for more than 20 years. Possibly, the B allele has a

Figure 1. Electropherogram of the IGF-I gene fragment digested with *SnaB*I and submitted to electrophoresis on 3.5% agarose gel.



Lane 1, 1-kb Plus DNA Ladder as molecular weight marker; lane 2, genotype BB (249-bp band); lanes 3 and 4, genotype AB (bands of 226 and 249 bp). The 23-bp fragment is undetectable.

positive influence on the growth traits selected. This hypothesis is supported by the physiological role of IGF-I as a mediator of the effects of growth hormone, which plays an essential role in the growth and metabolism of mammals (Schreibman *et al*., 1993). Another aspect is that the identification of one genotype or one allele of a gene does not explain the full expression of a trait because it can be influenced by the expression of additional genes on the same or on other chromosomes, a fact indicating quantitative trait loci (QTL).

According to Machado *et al*. (2003), some QTLs that influence growth and carcass traits are located in the same chromosome region as the IGF-I gene and, due to its fundamental role in cell differentiation and tissue development, this gene may influence the effect of these QTLs. In a study on the effects of growth hormone and IGF-I gene polymorphisms on growth traits in the Canchim breed, Pereira *et al*. (2005) observed a significant effect of the IGF-I genotypes on birth and yearling weights in these animals.

RFLP-*Hae*III analysis of the PRL gene showed the presence of only one migration pattern, characterizing a monomorphism in the region studied. This high conservation may be significant by indicating that the regulation of this gene is under extremely rigid control by transcription factors, with high conservation being necessary for full activation. One fact that might explain the lack of variation in the 5'UTR PCR-RFLP *Hae*III region is the presence of binding sites for sugars, proteins and other molecules at this site of the DNA chain that possibly influence the process of DNA transcription. These regions are characterized by low mutation rates during evolution due to their importance for the maintenance of protein synthesis.

A Blast-n (http://www.ncbi.nlm.nih.gov) search was performed using the IGF-I and PRL promoter region sequences obtained in this study to determine their similarity to other mammalian species. A high similarity ranging from 96% to 99% was observed between the sequences obtained for the two genes and the *Bos taurus* sequences available in GenBank (AF017143 and AB098480). The nucleotide sequences of the IGF-I and PRL genes obtained in this study were deposited GenBank under the accession numbers DQ4998876 and DQ499887, respectively.

Analysis of the IGF-I sequences showed that the difference between alleles detected by PCR-RFLP was due to a single nitrogen base change from cytosine (allele B) to thymine (allele A) within the *SnaB*I restriction site. This point mutation occurred at nucleotide position 221 in the 5'-3' sequence of the amplified fragment and characterized the

Figure 2. Part of the IGF-I gene sequence amplified.

The upper sequence is derived from an animal with the BB genotype and the lower sequence is from an animal with the AB genotype. The complementary sequences of reverse primers are shown in gray and the arrow indicates the change of the nitrogen base cytosine (C) to thymine (T) at nucleotide position 221 in the 5′-3′ sequence of the amplified fragment.

*Sna*BI site (Figure 2). It should be emphasized that the reverse IGF-I primer used for amplification of the IGF-I gene fragment introduces part of this restriction site (ATGCA), which only becomes effective when the point mutation mentioned above occurs, i.e., a change from C to T. The results obtained agree with those reported by Ge *et al*. (2001), who detected the same base change in Angus animals. These authors first identified this polymorphism by SSCP analysis.

Sequencing of the IGF-I gene fragment revealed nucleotide sequence variations other than those identified by RFLP analysis. These variations were identified at nucleotide position 22-23 (change of TC to CG), 60-62 (TCC to CAG), and 93 (C to A) in the 5'-3' direction of the fragment analyzed, and may serve as a basis for further studies of bovine molecular markers. Furthermore, the present sequencing analysis may contribute to molecular studies involving *Bos indicus* breeds since most sequences deposited in GenBank are derived from *Bos taurus*.

Conclusions

Under the present experimental conditions, the PRL and IGF-I gene polymorphisms were not informative markers for sexual precocity since only one allele of either locus was observed in the groups studied. Thus, the study of other regions in the IGF-I and PRL genes might provide molecular data that can be used in the future for the selection of sexually precocious animals. Sequencing permitted the identification of nucleotide sequence variations in the 5'-flanking region of the IGF-I gene not detected by PCR-RFLP, which may serve as a basis for studies of molecular markers in cattle.

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