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Growth hormone mRNA expression in the pituitary of *Bos indicus* and *Bos taurus* x *Bos indicus* crossbred young bulls treated with recombinant bovine somatotropin

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ABSTRACT. The effects of breed and of recombinant bovine somatotropin (rbST) treatment on growth hormone gene expression were studied in young bulls. The experiment was completely randomized in a [2 x 2]-factorial arrangement, using two levels of rbST (0 or 250 mg/animal/14 days), and two breed groups (Nelore and Simmental x Nelore crossbred). A cDNA encoding *Bos indicus* growth hormone was cloned and sequenced for use as a probe in Northern and dot blot analyses. Compared to the *Bos taurus* structural gene, the *Bos indicus* cDNA was found to begin 21 bases downstream from the transcription initiation site and had only two discrepancies (C to T at position 144-His and T to C at position 354-Phe), without changes in the polypeptide sequence. However, two amino acid substitutions were found for *Bubalus* spp., which belong to the same tribe. The rbST treatment did not change any of the characteristics evaluated (body and pituitary gland weights, growth

hormone mRNA expression level). Crossbred animals had significantly higher body weight and heavier pituitaries than Nelore cattle. Pituitary weight was proportional to body weight in both breed groups. Growth hormone mRNA expression in the pituitary was similar (P>0.075) for both breed and hormonal treatment groups, but was 31.9% higher in the pure Nelore group, suggesting that growth hormone gene transcription regulation differs among these breeds.

Key words: Recombinant bovine somatotropin, Bovine pituitary Growth hormone gene expression, *Bos indicus*, *Bos taurus*

INTRODUCTION

Endocrine growth control involves complex interactions of several hormones and growth factors, acting in both an endocrine and a paracrine or autocrine manner. In this context the somatotropic axis, which is composed of the pituitary growth hormone (GH or somatotropin), its receptor (GHR), insulin-like growth factors (IGF), IGF binding proteins (IGFBP) and the IGF-I receptor (IGF-IR), plays an essential role in postnatal growth regulation, especially in nutrient utilization. Although GH blood levels depend on physiological states and other regulatory factors, there is evidence of an association of genetic characteristics with GH plasma levels. Increased GH blood levels have been reported in dairy cattle selected for elevated milk yield (Peel and Bauman, 1987), lean-selected sheep (Venters et al., 1995, cited by Fleming et al., 1997) and low backfat pigs (Althen and Gerritz, 1976), compared with divergently selected lineages. GH gene polymorphism affects GH plasma levels (Schlee et al., 1994). However, elevated GH plasmatic levels are apparently not dependent on GH gene transcription activity, since Fleming et al. (1997) found higher total GH in lean than in fat lamb pituitary glands, though GH mRNA concentrations were similar. Recombinant bovine somatotropin (rbST) treatment of ruminants with an intact somatotropic axis may also alter pituitary GH production. Moseley et al. (1992) observed an anterior pituitary gland weight increase in beef steers treated with rbST, but a reduction in GH content and concentration in this gland. We evaluated GH mRNA expression in young bull pituitaries from two different breed groups treated with rbST.

MATERIAL AND METHODS

Animals and experimental procedures

Twenty intact male calves were weaned at 7-8 months and housed in collective pens for 180 days. A completely randomized experimental design was applied in a 2 x 2-factorial arrangement, using two levels of rbST (0 or 250 mg/animal/14 days) and two breed groups (Nelore and Simmental x Nelore crossbred). Lactropin-500® (500 mg) was supplied in syringes in a prolonged release vehicle formulated by Monsanto Agricultural Co. (St. Louis, MO, USA). Nontreated animals were injected with saline. Diet, designed for a weight gain of 1.0 kg/animal/day, was based on corn silage and concentrate (cracked corn, whole soybeans, urea and mineral

premix). At 13-14 months, 10 young bulls of each breed group (five treated and five nontreated) were slaughtered and their pituitary glands were collected, weighed and immediately frozen in liquid nitrogen.

Cloning and sequencing of GH cDNA

Total RNA was isolated from Nelore (*Bos indicus*) anterior pituitary glands by the acidified phenol-guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Polyadenylated RNA (poli A^+) was isolated from 1.83 mg of this RNA by oligo-dT-cellulose chromatography, as described by Sambrook et al. (1989). The polyadenylated RNA was transcribed into single-strand complementary DNA using reverse transcriptase and the second DNA strand synthesis was done using DNA polymerase I, as described in the ZAP-cDNA Synthesis kit (Stratagene). After packaging, lambda recombinants were transfected into an *Escherichia coli* cell line (XL1-blue MRF') and the amplified library was screened using a partial GH bovine cDNA (kindly provided by Dr. W. Rodrigues, University of São Paulo, Ribeirão Preto Campus) labeled by the random-priming method using [α-^{32}P]-dATP and [α-^{32}P]-dCTP. Of 8,500 clones, 420 were hybridized in the first screening, and 20 of which were chosen from the second screening for digestion with *Xho*I and *Eco*RI. After separation on 1.0% agarose gel, fragment sizes were determined by Southern blot (Sambrook et al., 1989), with 14 clones showing insertion in the 500- to 800-pb range. A cDNA clone, GH35, containing the largest insert, was fully sequenced as double-stranded DNA by the dideoxy-mediated chain termination method (Sanger et al., 1977) using a cycle sequencing kit (Pharmacia) and [α-^{32}P]-dATP. Initially, pB SK (+) T3 and T7 primers (Stratagene) were used to sequence the 5' and 3' ends, followed by complete sequencing of both strands by primer walking. The *Bos indicus* GH cDNA was used as a probe in GH mRNA measurements and for comparison with *Bos taurus* and *Bubalus bubalis* sequences.

Northern blotting

Total anterior pituitary RNA and poli A^+ mRNA were denatured by formamide and formaldehyde and separated according to size on a 1.5% denaturing agarose gel (Sambrook et al., 1989). After electrophoresis, RNA was transferred by capillarity to a nylon membrane (Zeta-Probe, BioRad) and then probed with GH cDNA (clone GH35) labeled with [α-^{32}P]-dCTP under standard conditions (Sambrook et al., 1989).

Cloning of a genomic sequence of rat 28S rRNA

Genomic DNA from rat liver isolated according to Sambrook et al. (1989) was used as a template in a PCR with a forward (28Sup; 5'-GTGAGGCGGGGGGGCGA-3') and a reverse primer (28Sdown; 5'-TGACGGGCGAGAGGGCG-3') to amplify a 779-bp fragment corresponding to bases 3803 to 4581 from rat 28S rRNA gene sequence (Chan et al., 1983), which encompasses a 585-*Sac*I/*Ava*I restriction fragment. PCR was carried out in a 25-µl volume in an MJ Research Thermal Cycler using a single-initial denaturation cycle at 95ºC for 3 min, annealing at 58ºC for 1 min and extension at 72ºC for 1 min; then, 35 cycles of denaturation at 94ºC for 30 s, annealing at 58ºC for 30 s and extension at 72ºC for 45 s, and lastly, extension at

72ºC for 5 min. Reaction mixture concentrations were: 1X Taq Buffer (Pharmacia), 2.5 mM dNTPs, 0.4 µM of each primer, 0.5% formamide, 0.5 U Taq DNA polymerase (Pharmacia), and 100 ng genomic DNA. The fragment was visualized on a 1.0% agarose gel stained with ethidium bromide and purified from the gel (Sambrook et al., 1989). After digestion with *Sac*I and *Ava*I, the 585-bp fragment was isolated from a 1.0% agarose gel and cloned into a pUC18 plasmid previously digested with the same enzymes. Following transformation in *E. coli* strain DH5α, plasmid DNA from a large-scale preparation was purified by cesium chloride gradient (Sambrook et al., 1989) and double-digested with *Sac*I and *Ava*I restriction enzymes. The 585-bp fragment was isolated from agarose gel and used as a probe in the dot-blot hybridization.

RNA quantification by dot blotting

Total RNA was extracted from whole pituitary using the acidified phenol-guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) and the concentration determined by measuring absorbance at 260 nm (Sambrook et al., 1989). The integrity and DNA contamination of RNA extracted from each pituitary was evaluated by running an agarose gel electrophoresis under denaturating conditions and protein contamination was examined by the A_{260}/A_{280} nm ratio (Sambrook et al., 1989). If the A_{260}/A_{280} nm ratio was lower than 1.8, a new phenol-chloroform extraction was done. Growth hormone mRNA was quantified by the dot-blot hybridization method using a Bio-Dot® microfiltration apparatus (Bio-Rad) according to manufacturer instructions. Total RNA (1.25 and 0.625 ng) was blotted onto Hybond-N® nylon membrane (Amersham) and fixed by UV crosslinking (160,000 µJ/cm^2) using a CL-1000 ultraviolet crosslinker (UVP). Blots were hybridized with bovine GH cDNA labeled to high specific activity with [α-^{32}P]-dATP and [α-^{32}P]-dCTP by the random-priming method (Feinberg and Vogelstein, 1983) in a hybridization oven (Hybaid) and exposed to Kodak T-MAT X-ray film at -80ºC after washing in standard conditions. After exposure, the membrane was washed for 20 min in boiling water to remove GH cDNA probe and re-exposed to X-ray film to assure total removal of the hybridization signal. The membrane was then reprobed with a genomic sequence of 28S rRNA (28S rDNA) in order to correct for possible differences in quantifying and pipetting RNA samples. Signals were measured with a densitometer (CS-9301, Shimadzu) at 550 nm and in the zigzag-scanning mode. The area of the peaks was converted to ng of GH mRNA by using a standard curve constructed with different concentrations of GH cDNA blotted on the same membrane.

RESULTS

Growth hormone cDNA

The complete DNA sequence and the encoded protein of the GH35 clone, the longest clone isolated from the cDNA library, was determined (Figure 1). The cloned cDNA contained 821 bp including 42 bp in the 5'-untranslated region and 576 bp coding the entire pre-hormone, the total 3'-untranslated region (101 bp) and a poly(A) tail with 24 bp. This sequence corresponds to complete exons 2, 3, 4 and 5 of the *Bos taurus* GH gene (Gordon et al., 1983) with the 5'-untranslated region starting at base 21 of the first exon. In the Northern analysis (Figure 2), a single-GH mRNA transcript of approximately 1,000 bp was detected, similar to findings obtained by Hampson and Rottman (1987). This value exceeds the 821 bp of the isolated clone,

-42	gtt	cac	cag	acg	act	cag	ggt	cct	gtg	gac	agc	tca	cca	gct	ATG	ATG	GCT	GCA
															MET	MET	Ala	Ala
13	GGC	CCC	CGG	ACC	TCC	CTG	CTC	CTG	GCT	TTC	GCC	CTG	CTC	TGC	CTG	CCC	TGG	ACT
	Gly	Pro	Arg	Thr	Ser	Leu	Leu	Leu	Ala	Phe	Ala	Leu	Leu	Cys	Leu	Pro	Trp	Thr
67	CAG	GTG	GTG	GGC	**GCC**	TTC	CCA	GCC	ATG	TCC	TTG	TCC	GGC	CTG	TTT	GCC	AAC	GCT
	Gln	Val	Val	Gly	Ala	Phe	Pro	Ala	MET	Ser	Leu	Ser	Gly	Leu	Phe	Ala	Asn	Ala
121	GTG	CTC	CGG	GCT	CAG	CAC	CTG	CAC	CAG	CTG	GCT	GCT	GAC	ACC	TTC	AAA	GAG	TTT
	Val	Leu	Arg	Ala	Gln	His	Leu	His	Gln	Leu	Ala	Ala	Asp	Thr	Phe	Lys	Glu	Phe
175	GAG	CGC	ACC	TAC	ATC	CCG	GAG	GGA	CAG	AGA	TAC	TCC	ATC	CAG	AAC	ACC	CAG	GTT
	Glu	Arg	Thr	Tyr	Ile	Pro	Glu	Gly	Gln	Arg	Tyr	Ser	Ile	Gln	Asn	Thr	Gln	Val
229	GCC	TTC	TGC	TTC	TCT	GAA	ACC	ATC	CCG	GCC	CCC	ACG	GGC	AAG	AAT	GAG	GCC	CAG
	Ala	Phe	Cys	Phe	Ser	Glu	Thr	Ile	Pro	Ala	Pro	Thr	Gly	Lys	Asn	Glu	Ala	Gln
283	CAG	AAA	TCA	GAC	TTG	GAG	CTG	CTT	CGC	ATC	TCA	CTG	CTC	CTC	ATC	CAG	TCG	TGG
	Gln	Lys	Ser	Asp	Leu	Glu	Leu	Leu	Arg	Ile	Ser	Leu	Leu	Leu	Ile	Gln	Ser	Trp
337	CTT	GGG	CCC	CTG	CAG	TTT	CTC	AGC	AGA	GTC	TTC	ACC	AAC	AGC	TTG	GTG	TTT	GGC
	Leu	Gly	Pro	Leu	Gln	Phe	Leu	Ser	Arg	Val	Phe	Thr	Asn	Ser	Leu	Val	Phe	Gly
391	ACC	TCG	GAC	CGT	GTC	TAT	GAG	AAG	CTG	AAG	GAC	CTG	GAG	GAA	GGC	ATC	CTG	GCC
	Thr	Ser	Asp	Arg	Val	Tyr	Glu	Lys	Leu	Lys	Asp	Leu	Glu	Glu	Gly	Ile	Leu	Ala
445	CTG	ATG	CGG	GAG	CTG	GAA	GAT	GGC	ACC	CCC	CGG	GCT	GGG	CAG	ATC	CTC	AAG	CAG
	Leu	MET	Arg	Glu	Leu	Glu	Asp	Gly	Thr	Pro	Arg	Ala	Gly	Gln	Ile	Leu	Lys	Gln
499	ACC	TAT	GAC	AAA	TTT	GAC	ACA	AAC	ATG	CGC	AGT	GAC	GAC	GCG	CTG	CTC	AAG	AAC
	Thr	Tyr	Asp	Lys	Phe	Asp	Thr	Asn	MET	Arg	Ser	Asp	Asp	Ala	Leu	Leu	Lys	Asn
553	TAC	GGT	CTG	CTC	TCC	TGC	TTC	CGG	AAG	GAC	CTG	CAT	AAG	ACG	GAG	ACG	TAC	CTG
	Tyr	Gly	Leu	Leu	Ser	Cys	Phe	Arg	Lys	Asp	Leu	His	Lys	Thr	Glu	Thr	Tyr	Leu
607	AGG	GTC	ATG	AAG	TGC	CGC	CGC	TTC	GGG	GAG	GCC	AGC	TGT	GCC	TTC	TAG	ttg	cca
	Arg	Val	MET	Lys	Cys	Arg	Arg	Phe	Gly	Glu	Ala	Ser	Cys	Ala	Phe	STP		
661	gcc	atc	tgt	tgt	ttg	ccc	ctc	ccc	cgt	gcc	ttc	ctt	gac	cct	gga	agg	tgc	cac
715	tcc	cac	tgt	cct	ttc	cta	ata	aaa	tga	gga	aat	tgc	atc	gc				

Figure 1. Complete sequence of the cDNA encoding the bovine growth pre-hormone. The deduced amino acid sequence is indicated and the pre-sequence is underlined. The first amino acid of the mature hormone (Ala) is marked in bold. This sequence is deposited at GenBank (accession number AF034386).

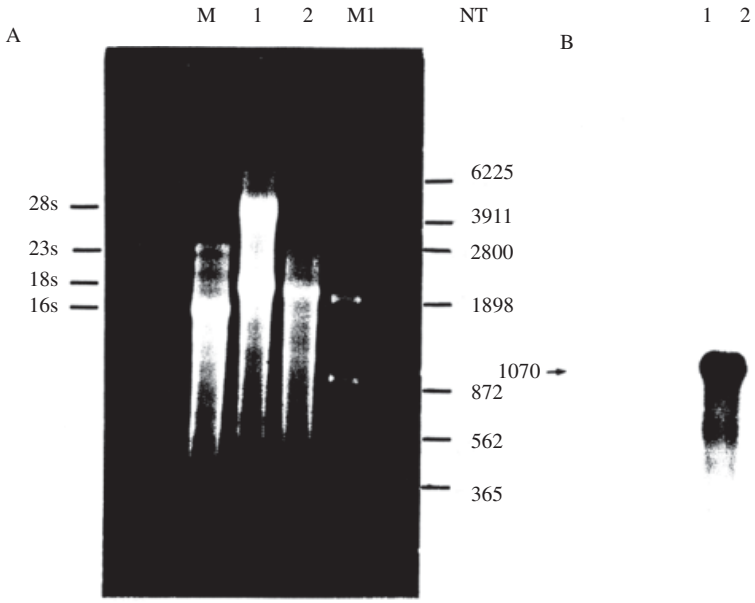

Figure 2. Northern blot of total RNA and poly(A)-rich mRNA from Nelore (*Bos indicus*) anterior pituitary. **A** - Electrophoresis on a 1.0% agarose gel stained with ethidium bromide. M: 8 µg of 16S and 23S *Escherichia coli* rRNA (Sigma Chemical Co.); lane 1: 21.9 µg of total RNA; lane 2: 9 µg of poly(A$^+$) mRNA; M1: RNA size markers (Promega). Marker size is indicated in nucleotides (NT). **B** - Blot on nylon membrane of the agarose gel shown in *A*, hybridized with [^{32}P]-labeled GH cDNA. Conditions were as described in Material and Methods and labels are the same as for *A*.

indicating that the latter is not a full-length cDNA, though it contains most sequences present in the mRNA. Comparing the cDNA with the gene sequence yielded only two discrepancies, both in the coding region, without changing encoded amino acids (C to T at position 144-His and T to C at position 354-Phe). There was no difference in the amino acid sequence compared to the two published sequences of *Bos taurus* GH mRNA (Miller et al., 1980; Seeburg et al., 1983), while three discrepancies in the nucleotide sequence were found. On the other hand, there were nine differences at the nucleotide level in the cDNA-coding region in comparison to the *Bubalus arnee bubalus* sequence (GenBank accession number AJ011533), with two amino acid changes (G9S and R17W), while comparison with the *Bubalus arnee* sequence (GenBank accession number X72947) gave 15 nucleotide level differences with two amino acid changes (A12T and G130V).

Growth hormone mRNA quantitative analysis

Dot-blot hybridization was used to analyze the GH expression in young bull pituitary glands from two different breed groups treated and nontreated with rbST. To determine the ability of the dot-blot assay to accurately measure GH mRNA levels, different amounts of GH cDNA and total RNA from pituitaries were blotted onto nylon membranes and probed with GH cDNA (Figure 3, panel A). When reprobed with the 28S rDNA, the sample signals in the membrane

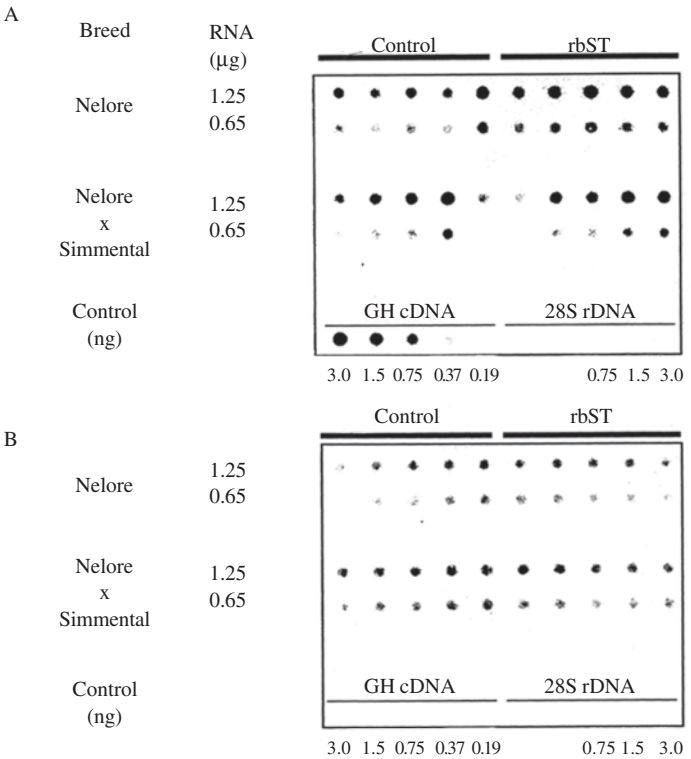

Figure 3. Dot-blot quantification of bovine pituitary GH mRNA. Two different amounts of total RNA (1.25 and 0.65 µg) were blotted onto nylon membranes. RNA from different animals was blotted from left to right and the two different RNA amounts are from top to bottom. **A** - Membrane hybridized with GH cDNA labeled with ^{32}P. To construct the standard curve for GH mRNA quantification different amounts of GH cDNA (3.0, 1.5, 0.75, 0.375, and 0.187 ng) were blotted on the left side of the membrane. The GAPDH cDNA (3.0, 1.5, and 0.75 ng) blotted on the right side was used as a negative control. **B** - Membrane reprobed with DNA fragment of 28S rRNA (28S rDNA) labeled with ^{32}P.

were very similar (Figure 3, panel B). The linear ranges of detection were from 0.187 to 3.0 ng (Figure 4), with the signals from 0.65 µg of total RNA being within the standard curve for all the animals. Growth hormone mRNA levels were similar (P>0.075) in both breed groups, although GH mRNA levels in Nelore pituitaries were 32.3% higher than those of the Simmental x Nelore breed group (Table 1). Somatotropin administration did not affect the GH mRNA levels (Table 1).

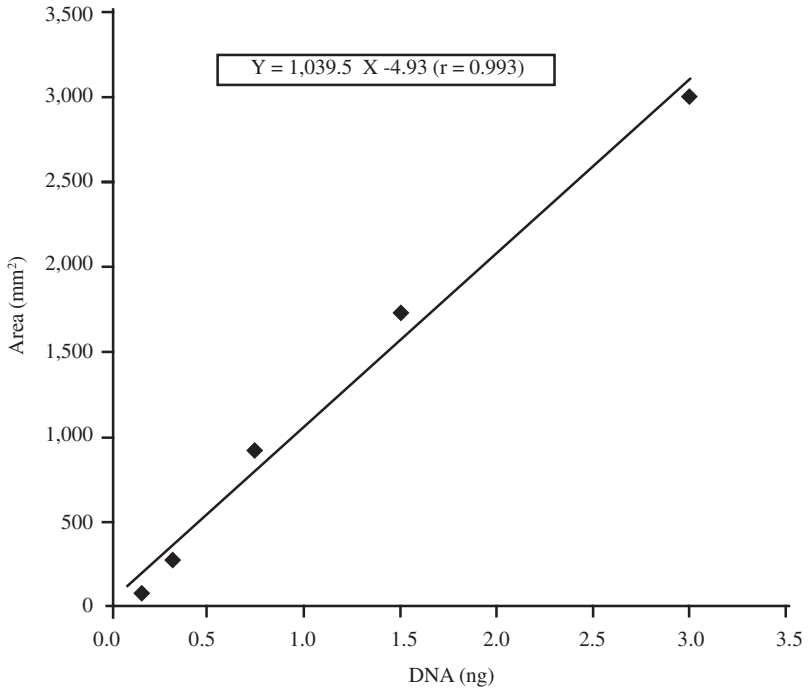

Figure 4. Standard curve for growth hormone (GH) mRNA quantification. Blots were analyzed by densitometry and a standard curve for GH mRNA quantification was obtained by plotting GH cDNA amounts (Figure 3B) against the respective peak areas.

Table 1. Body weight (kg), pituitary weight (g), relative pituitary weight as a percent of body weight (%BW) and growth hormone (GH), mRNA amounts (ng) in bovine breed groups and hormonal treatment groups.

Treatments	Live weight** (kg)	Pituitary* weight (g)	Pituitary^{ns} (%BW x 10^3)	GH mRNA^{ns} (ng/µg RNA)
Breed group				
Nelore	269.40 ± 27.6^a	1.10 ± 0.07^a	0.41 ± 0.01^a	2.58 ± 0.03^a
Sim-Nel	390.00 ± 21.9^b	1.59 ± 0.07^b	0.41 ± 0.00^a	1.95 ± 0.10^a
Hormone treatment				
rbST	327.30 ± 22.9^a	1.28 ± 0.06^a	0.39 ± 0.01^a	2.26 ± 0.04^a
Saline	332.10 ± 26.7^a	1.41 ± 0.07^a	0.42 ± 0.01^a	2.27 ± 0.16^a

Sim-Nel = Simmental-Nelore
rbST = recombinant bovine somatotropin
ns - nonsignificant difference
*P<0.05.
**P<0.001.
Different lower case letters indicate that means are different by the Tukey test (P<0.05).

Simmental x Nelore breed group animals had significantly higher body weights (P<0.01) and heavier pituitary glands (P<0.05) than Nelore cattle (Table 1). However, relative pituitary weight (as a percent of body weight) was similar in the two breed groups (Table 1). Somatotropin administration did not affect body weight, pituitary weight or relative pituitary weight (Table 1).

DISCUSSION

At the same age and under identical management conditions, Simmental x Nelore crossbreeds had significantly (P<0.05) higher body weight and heavier pituitaries than Nelore cattle, but pituitary weight was proportional to body weight in both breed groups (Table 1). In spite of a discrepancy in growth rate between the two breed groups, it appears that pituitary growth occurs allometrically. However, pituitary weight differences may result from genetic selection. Fleming et al. (1997) reported heavier pituitaries in a lean-selected sheep line with a high body weight than in a fat-selected line, though pituitary size differences were body-weight independent. Higher pituitary weights have also been reported in swine lineages selected for low backfat (Althen et al., 1976). Besides higher pituitary weights, the authors of both of these papers reported higher plasmatic GH levels in both lean-selected sheep and low-backfat selected swine. Pituitary weight differences result from an increase in cell number rather than in cell size (Venters et al., 1995, cited by Fleming et al., 1997), suggesting an increase in somatotroph number, causing higher GH production.

In our study, rbST treatment had no significant effect on GH mRNA transcription or on pituitary weights. Moseley et al. (1992) reported a pituitary weight increase associated with GH content reduction when finishing steers received daily rbST supplements. Although rbST in a prolonged release vehicle can mimic daily hormonal injections and can sustain an elevated basal plasma GH concentration for nearly eight days after its application (Slaba et al., 1994), recombinant hormone dose and intervalic administration did not affect pituitary GH expression in our experiment.

Pituitary GH production and GH plasmatic levels were not evaluated in the present study, but plasmatic IGF-I dosed before rbST administration was significantly (P<0.05) higher in the crossbred cattle (data not shown), possibly due to the higher plasmatic GH levels in this group, as reported by Breier and Sauerwein (1995).

Since the Nelore animals had a lower growth rate and lower plasmatic IGF-I levels, the tendency towards higher GH mRNA concentrations was unexpected, but it could involve polymorphisms in the GH gene sequence.

Relationships between gene variants and plasmatic GH and IGF-I levels have been reported by Schlee et al. (1994), who observed that Holstein cows homozygous for the leucine variant of the GH protein have higher GH plasma levels than their heterozygous counterparts. On the other hand, the heterozygous genotype in males was associated with higher IGF-I plasmatic levels and a higher genetic value for daily weight gain than was the homozygous genotype. However, animals heterozygous for this variant have not been identified in Nelore populations (Rosa, 1997; Tambasco, 1998; Mendes, 2000), suggesting that the leucine allele is predominant over the valine allele in this breed.

Sequence variations of the region involved in bovine GH gene regulation have also been reported, with Hecht and Geldermann (1996) identifying six variable nucleotide sites in the 5'-flanking region, some of them potentially binding sites for trans-acting factors possibly

involved in genetic expression. Rodrigues et al. (1998) identified an AAG deletion nine nucleotides upstream from TATA box in beef but not in dairy cattle. This allele's frequency was 0.15 in 120 Nelore animals. In our study, *Bos indicus* cDNA showed only two discrepancies when aligned with the complete bovine GH gene sequence. However, no change was found in the polypeptide amino acid sequence, regardless of genetic differences found between *Bos taurus* and *Bos indicus* in protein (Manwell and Baker, 1980) and chromosome Y (Kieffer and Cartwright, 1968) polymorphisms. However, two amino acid substitutions were found for *Bubalus* spp., which belong to the same tribe.

In conclusion, pituitary weight was proportional to body weight in both breed groups and the amount of GH mRNA was slightly but not significantly higher in *Bos indicus* than in *Bos taurus* crosses. The rbST treatment had no effect on GH gene expression in the pituitary gland, even though it was applied in a sustained release vehicle.

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