Sequence Variations in the Bovine Growth Hormone Gene Characterized by Single-Strand Conformation Polymorphism (SSCP) Analysis and Their Association with Milk Production Traits in Holsteins

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ABSTRACT

Sequence variations in the bovine growth hormone (GH) gene were investigated by single strand conformation polymorphism (SSCP) analysis of seven amplified fragments covering almost the entire gene (2.7 kb). SSCPs were detected in four of these fragments and a total of six polymorphisms were found in a sample of 128 Holstein bulls. Two polymorphisms, a T→C transition in the third intron (designated GH4.1) and an A→C transversion in the fifth exon (designated GH6.2), were shown to be associated with milk production traits. *GH4.1*^{c}/*GH4.1*^{c} bulls had higher milk yield than *GH4.1*^{c}/*GH4.1*^{t} ($P \leq 0.005$) and *GH4.1*^{t}/*GH4.1*^{t} ($P \leq 0.0022$) bulls. *GH4.1*^{c}/*GH4.1*^{c} bulls had higher kg fat ($P \leq 0.0076$) and protein ($P \leq 0.0018$) than *GH4.1*^{c}/*GH4.1*^{t} bulls. Similar effects on milk production traits with the GH6.2 polymorphism were observed with the *GH6.2*^{a} allele being the favorable allele. The average effects of the gene substitution for GH4.1 and GH6.2 are similar, with ±300 kg for milk yield, ±8 kg for fat content and ±7 kg for protein content per lactation. The positive association of *GH4.1*^{c} and *GH6.2*^{a} with milk production traits may be useful for improving milk performance in dairy cattle.

IN dairy cattle, the primary focus of selection, at least in North America, has been to improve milk yield, and tremendous gains have been achieved using classical quantitative genetics. Although the genes that affect a polygenic trait such as milk production are unknown, a number of potential candidate genes have been recognized. Candidate genes are selected on the basis of known relationship between physiological or biochemical processes and a trait and are tested as putative quantitative trait loci (QTL). Current knowledge in dairy biology indicates that genetically superior animals differ from lesser animals mainly in their regulation of nutrient utilization and that growth hormone (GH) exerts a key control in nutrient use (BAUMAN 1992), mammary development (SEJRSEN *et al.* 1986), and growth (BRIER *et al.* 1991) and also modulates intermediary metabolism and other physiological processes *e.g.*, aging (COPRAS *et al.* 1993) and immune responsiveness (BLALOCK 1994). Thus the GH gene is a promising candidate gene worth studying for its effects on milk- and growth-related and immune response traits. Selection for milk yield has been shown to be associated with increased blood levels of GH (PEEL and BAUMAN 1987; BRONCZEK *et al.* 1988; LUKES *et al.* 1989). This suggests that variations in the levels of GH could be used as an indicator for potential milk yield and thus be incorporated into a selection index. However, blood levels of GH are dependent on physiological states of the animal and vary with the stage of lactation or even the time of day. For this reason, estimating breeding value on the basis of GH levels would require multiple measurements over the course of several lactations.

There is evidence for an association of genetic variants of the GH gene with plasma levels of GH (SCHLEE *et al.* 1994), suggesting that at least some variations of the GH levels are caused by mutations in the GH gene itself. Identification of such mutations would permit selection at the DNA level without necessitating measurement of GH levels. In order to detect such mutations, we have used single-strand conformation polymorphism (SSCP) analysis (ORITA *et al.* 1989a,b), a technique based on the principle that single-stranded DNA molecules form specific sequence-based secondary structures under nondenaturing conditions. Our objective was to screened the entire length of the bovine GH gene for sequence variations and study the associations of changes in potential GH sequence variants with changes in milk production traits in Holsteins.

MATERIALS AND METHODS

DNA samples: Semen samples from 128 Holstein bulls representing bulls used for artificial insemination from 1950–1987 were obtained from the Centre d'insemination artificielle du Quebec (Saint-Hyacinthe, Quebec, Canada), the Western Ontario Breeders Inc. (Woodstock, Ontario, Canada) and the United Breeder Inc. (Guelph, Ontario, Canada). Genomic DNA was extracted as previously described (ZADWORNY and KUHNLEIN 1990).

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TABLE 1

Sequence and position of oligonucleotide primers

Fragment	Primer	Primer sequence	Location	Size (bp)
GH1	P1	5′-GGTGGGTTGCCTTTCTCTTCT-3′	8–471	464
	P2	5′-TGTCATCATCCCGTCTCCACT-3′		
GH2	P3	5′-TCTCAAGCTGAGACCCTGTGT-3′	408–860	453
	P4	5′-GGCCAAATGTCTGGGTGTAGA-3′		
GH3	P5	5′-TTGGGCTTTAGGGCTTCCGAA-3′	805–1224	420
	P6	5′-TGAACTCCTCAGTTTCCTCCC-3′		
GH4	P7	5′-GGACAGAGATACTCCATCCAG-3′	1380–1724	345
	P8	5′-AGATGCGAAGCAGCTCCAAGT-3′		
GH5	P9	5′-TTGGAGCTGCTTCGCATCTCA-3′	1706–2071	366
	P10	5′-ATTTTCCACCCTCCCCTACAG-3′		
GH6	P11	5′-TAGGGGAGGGTGGAAAATGGA-3′	2054–2457	404
	P12	5′-GACACCTACTCAGACAATGCG-3′		
GH7	P13	5′-CACTCCCACTGTCCTTTCCTA-3′	2396–2850	455
	P14	5′-ACTTCCTCACATGTTGGAGGC-3′		

Based on the nucleotide sequence of bovine GH gene from GORDON *et al.* (1983).

DNA amplification with polymerase chain reaction (PCR): Based on the published nucleotide sequence information of the bovine GH gene (GORDON *et al.* 1983), pairs of oligonucleotide primers were synthesized to amplify seven GH fragments (designated GH1, GH2, GH3, GH4, GH5, GH6 and GH7). The gene from −641 bp upstream of the first exon to 411 bp downstream of the last exon, except a 155-bp fragment from position 1225 to 1379 was analyzed for each bull. The primer sequences, location and size of the amplified fragments are shown (Table 1). PCR was performed in a reaction volume of 25 μl using 100 ng of DNA, 0.5 μM of each primer, 1× PCR buffer [10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$ and 50 mM KCl], 5% deionized formamide, 200 μM dNTP and 0.625 units of *Thermus thermophilus* (Tth) DNA polymerase (Pharmacia). The amplification was carried out for 35 cycles at 92° × 30 sec, 59° × 80 sec and 72° × 90 sec using a DNA thermal cycler (Perkin Elmer Cetus Corp.).

SSCP analysis: SSCP was carried out with a Bio-Rad "Mini-Protein II" (Bio-Rad) vertical gel. One microliter of the PCR product was diluted with 15 μl of a solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The mixture was then denatured at 100° for 5 min, cooled in ice for 5 min and loaded on a nondenaturing 12–20% acrylamide:bis-acrylamide (49:1) gel. Electrophoresis was performed in 1× Tris borate (pH 8.3)-EDTA buffer at 10–12.5 volts/cm for 6–24 hr at room temperature. DNA was detected by silver staining.

Sequence analysis: DNA fragments that displayed a modified electrophoretic pattern were selected for sequencing. For blunt-end ligation into plasmid vectors, the amplified PCR products were concentrated by ethanol precipitation and subjected to electrophoresis in a 1% agarose gel. The DNA was isolated from the gel using DEAE cellulose membrane (SAMBROOK *et al.* 1989), phosphorylated at the 5′-terminus by T4 polynucleotide kinase (Pharmacia) and ligated into the *Sma*I site of pUC18 plasmid. DNA sequences were determined by the dideoxy-chain termination method of SANGER *et al.* (1977) with [^{35}S]dATP using a T7 sequencing kit (Pharmacia).

Production traits: All estimated breeding values were provided by the Canadian Holstein Breeders Association. The bull's breeding values for milk related traits (kg milk, kg fat and kg protein) were estimated with the best linear unbiased procedure (BLUP) based on an animal model with a relationship matrix. Preadjustments were made for effects of age and month of calving of daughters. The model included fixed effects of herd-year-season and age group of sires.

Statistical analysis: The effect of GH genotypes on the bull's estimated breeding values for kg milk, kg fat and kg

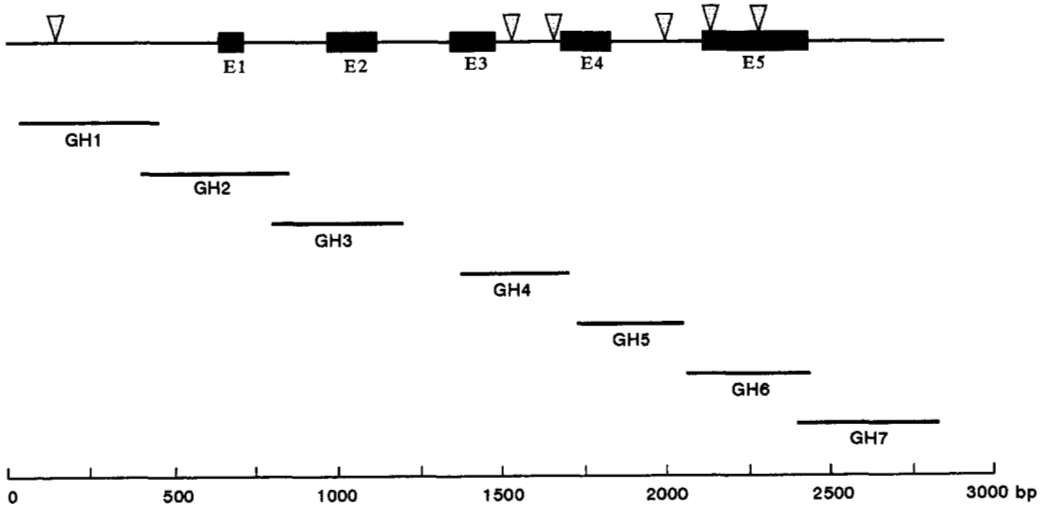

FIGURE 1.—Map of the bovine GH gene with the position and length of the GH fragments. Open arrowheads indicate the locations of the polymorphic sites.

TABLE 2

Optimal conditions for SSCP analysis of GH1 and GH4–GH6 fragments of the bovine GH gene

GH fragment	Gel percentage (%)	Voltage (v)	Running time (hr)
GH1	20	100	20
GH4	15	80	24
GH5	20	100	24
GH6	12	100	6

protein for each polymorphic site were analyzed using least squares methods. As the breeding values are the best available estimates of the additive genotype of the bulls, no environmental effects were included in the model. The effect of birth-year of the bulls were included in the model to account for genetic progress made in the bulls from the 1950s to the 1980s. The model used was as follows:

$$Y_{ijk} = \mu + Year_i + \alpha_j + e_{ijk}$$

where Y_{ijk} is the breeding value (kg milk, kg fat or kg protein) of the k^{th} bull; μ is the least square mean of the trait; $Year_i$ is the effect of the i^{th} birth-year of the bull (genetic trend); α_j is the effect of the j^{th} genotype (j = 1, 2, 3); and e_{ijk} is the random residual effect. Type III sum of squares were used to evaluate the effect of GH polymorphisms. Difference in milk related traits values exhibited by the genotypes were compared by least square means. Average additive gene substitution effects of the milk production traits were calculated for the alleles of the GH polymorphisms which have significant effects on the traits according to FALCONER (1989).

RESULTS

Seven GH fragments (GH1–GH7), which cover almost the entire length of the bovine GH gene, were amplified. The position and length of the fragments are shown in Figure 1. No SSCPs were detected in the GH2, GH3 and GH7 regions under various electrophoretic conditions (data not shown). However, analysis of the GH1, GH4, GH5 and GH6 fragments did reveal polymorphisms. The optimal conditions of SSCP analysis for the four polymorphic fragments are listed in Table 2.

Analysis of the 464-bp GH1 fragment revealed two SSCP alleles. The faster migrating band was designated as A_1, and the slower one as B_1 (Figure 2A). The heterozygous individual (A_1B_1) presented three distinct bands indicating that only one strand of an allele had a novel conformation with a different electrophoretic mobility. The other strand, possessing the complementary sequence change, did not have a new conformation to cause a mobility shift and continued to migrate with the corresponding strand of the other allele. Sequence analysis revealed that the difference in mobility was due to a deletion (A_1) or an insertion (B_1) of a TGC repeat at position between 125 and 142 (Figure 1), resulting in five TGC repeats for the A_1 allele and six TGC repeats for the B_1 allele (Figure 2B).

Figure 3A shows the SSCP pattern of the GH4 fragment of the gene. Three alleles were observed and designated as A_4, B_4 and C_4, respectively, on the basis of increasing distances in the gel. All possible genotypes, A_4A_4, B_4B_4, C_4C_4, A_4B_4, A_4C_4 and B_4C_4 were observed

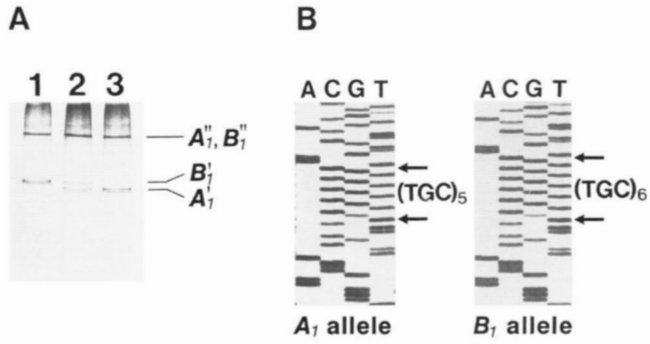

FIGURE 2.—Detection of a two-allele SSCP in the GH1 fragment of the bovine GH gene. (A) Banding pattern of GH1 SSCP. Lanes 1–3, individuals with genotypes B_1B_1, A_1B_1 and A_1A_1, respectively. [′] and [″] designate alternative single strands from the same allele. (B) Sequencing analysis of the GH1 fragment showing the deletion/insertion of a TGC repeat at position between 125 and 142. A_1 allele: deletion of TGC; B_1 allele: insertion of TGC.

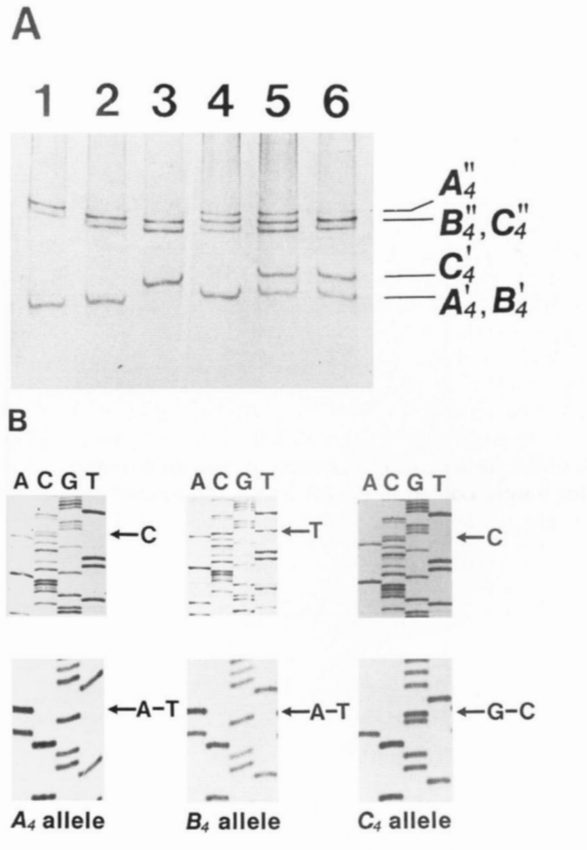

FIGURE 3.—Detection of a three-allele SSCP in the GH4 fragment of the bovine GH gene. (A) Banding pattern of GH4 SSCP. Lanes 1–6, individuals with genotypes A_4A_4, B_4B_4, C_4C_4, A_4B_4, A_4C_4 and B_4C_4, respectively. [′] and [″] designate alternative single strands from the same allele. (B) Sequencing analysis of the GH4 fragment showing the two mutations at positions 1547 (upper portion) and 1692 (lower portion, sequences shown are antisence strands). A_4: $C^{1547}...T^{1692}$; B_4: $T^{1547}...T^{1692}$; C_4: $C^{1574}...C^{1692}$.

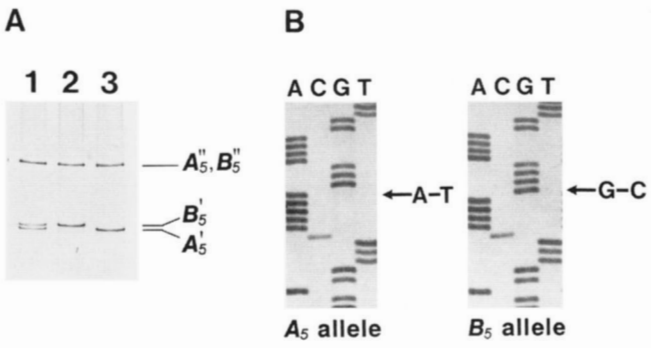

FIGURE 4.—Detection of a two-allele SSCP in the GH5 fragment of the bovine GH gene. (A) Banding pattern of GH5 SSCP. Lanes 1–3, individuals with genotypes A_5B_5, B_5B_5 and A_5A_5, respectively. [′] and [″] designate alternative single strands from the same allele. (B) Sequencing analysis of the GH5 fragment showing the mutation at position 2017 (sequences shown are antisence strands). A_5: T^{2017}; B_5: C^{2017}.

in the samples examined. For homozygous individuals (lanes 1, 2 and 3), three bands were observed under the SSCP conditions used. The two slowest migrating bands may represent two different conformations of the same strand. Sequence analysis of the region revealed two mutations in the third intron of the gene (Figure 1). Both mutations are T-to-C transitions with one at position 1547 and the other one at 1692 (Figure 3B). Three of the four possible combinations from the two mutations, $C^{1547}...T^{1692}$, $T^{1547}...T^{1692}$ and $C^{1547}...C^{1692}$, were observed, representing SSCP alleles A_4, B_4 and C_4, respectively. The polymorphisms in the GH fragment at the 1547 and 1692 mutation sites were designated GH4.1 and GH4.2, respectively.

The 366-bp GH5 region harbored one mutation at position 2017 as determined by sequence analysis. The mutation was a T-to-C transition which gave rise to two alleles (designated A_5 and B_5) detected by SSCP (Figure 4). As in the case of GH1, only three bands appeared in the heterozygous samples due to the same electrophoretic mobility of two of the four strands.

Figure 5A shows the SSCP results for the GH6 fragment. Three different alleles, A_6, B_6 and C_6, were identified and all the possible genotypes were observed. Sequence determination of the entire length of the fragment revealed two polymorphic sites at positions 2141 and 2291, respectively (Figure 5B). Both were base substitutions and located in the fifth exon (Figure 1). The mutation at position 2141 was a C-to-G transversion, while the mutation at 2291 was an A-to-C transversion. SSCP analysis detected three (A_6: $G^{2141}...A^{2291}$; B_6: $C^{2141}...A^{2291}$ and C_6: $C^{2141}...C^{2291}$) of the four possible combinations from the two mutations. The other possible combination ($G^{2141}...C^{2291}$) either does not produce unique band migration or does not exist in the sample population investigated. The polymorphisms in the GH6 fragment at positions 2141 and 2291 were designated GH6.1 and GH6.2, respectively. The sequence changes, nucleotide positions and allelic designation of the six polymorphisms in the bovine GH gene are presented in Table 3. In this table, alternative alleles of each polymorphism were assigned a superscript on the basis of the nucleotide present.

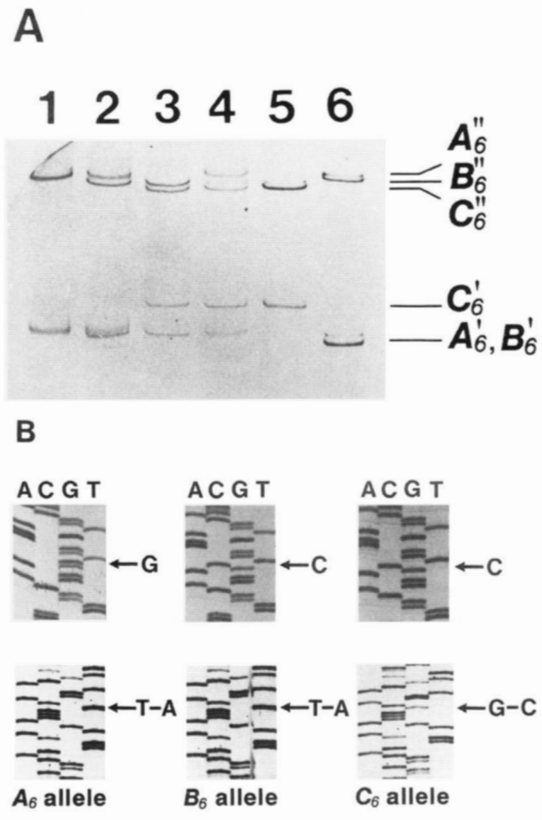

FIGURE 5.—Detection of a three-allele SSCP in the GH6 fragment of the bovine GH gene. (A) Banding pattern of GH6 SSCP. Lanes 1–6, individuals with genotypes A_6A_6, A_6B_6, A_6C_6, B_6B_6, B_6C_6 and C_6C_6, respectively. [′] and [″] designate alternative single strands from the same allele. (B) Sequencing analysis of the GH6 fragment showing the two mutations at positions 2141 (upper portion) and 2291 (lower portion, sequences shown are antisence strands). A_6: $G^{2141}...A^{2291}$; B_6: $C^{2141}...A^{2291}$; C_6: $C^{2141}...C^{2291}$.

The effects of the genotypes of the six GH polymorphisms on the breeding values for milk yield, fat content and protein content per lactation were examined in 128 Holstein bulls using least square methods. Least square means of the three GH genotypic classes of the six poly-

TABLE 3

Six polymorphisms in the bovine GH gene identified by SSCP analysis

Polymorphisms	Sequence change	Nucleotide position	Allele designation
GH1	del→ins TGC	125–140	$GH1^{d}$→$GH1^{i}$
GH4.1	C→T	1547	$GH4.1^{c}$→$GH4.1^{t}$
GH4.2	T→C	1692	$GH4.2^{t}$→$GH4.2^{c}$
GH5	T→C	2017	$GH5^{t}$→$GH5^{c}$
GH6.1	C→G	2141	$GH6.1^{c}$→$GH6.1^{g}$
GH6.2	A→C	2291	$GH6.2^{a}$→$GH6.2^{c}$

TABLE 4

Least square means of breeding values for milk yield, fat content and protein content in Holstein bulls with different GH genotypes

Polymorphism	GH Genotypes			*P* values
GH1	$GH1^d/GH1^d$	$GH1^d/GH1^i$	$GH1^i/GH1^i$	
	(n = 21)	(n = 68)	(n = 39)	
Milk	−372.5 ± 106.7	−419.7 ± 66.1	−458.4 ± 81.1	NS
Fat	−12.3 ± 3.6	−13.2 ± 2.2	−16.2 ± 2.7	NS
Protein	−12.4 ± 2.7	−14.6 ± 1.6	−14.2 ± 2.0	NS
GH4.1	$GH4.1^c/GH4.1^c$	$GH4.1^c/GH4.1^t$	$GH4.1^t/GH4.1^t$	
	(n = 95)	(n = 29)	(n = 4)	
Milk	−319.2 ± 56.3	−591.0 ± 82.3	−1033.7 ± 222.1	0.0006
Fat	−11.1 ± 2.0	−20.1 ± 2.9	−19.3 ± 7.8	0.0225
Protein	−11.5 ± 1.4	−19.2 ± 2.1	−21.0 ± 5.6	0.0038
GH4.2	$GH4.2^t/GH4.2^t$	$GH4.2^t/GH4.2^c$	$GH4.2^c/GH4.2^c$	
	(n = 86)	(n = 37)	(n = 5)	
Milk	−477.6 ± 59.3	−316.1 ± 82.1	−223.3 ± 197.1	NS
Fat	−14.6 ± 2.0	−12.8 ± 2.8	−9.8 ± 6.8	NS
Protein	−14.8 ± 1.5	−12.8 ± 2.1	−7.2 ± 4.9	NS
GH5	$GH5^t/GH5^t$	$GH5^t/GH5^c$	$GH5^c/GH5^c$	
	(n = 57)	(n = 55)	(n = 16)	
Milk	−476.1 ± 72.9	−385.2 ± 68.7	−360.7 ± 125.5	NS
Fat	−15.1 ± 2.5	−12.9 ± 2.3	−15.0 ± 4.2	NS
Protein	−14.2 ± 1.8	−14.3 ± 1.7	−11.8 ± 3.1	NS
GH6.1	$GH6.1^c/GH6.1^c$	$GH6.1^c/GH6.1^g$	$GH6.1^g/GH6.1^g$	
	(n = 106)	(n = 21)	(n = 1)	
Milk	−458.4 ± 55.6	−324.0 ± 108.5	578.9 ± 485.1	NS
Fat	−13.8 ± 1.9	−15.1 ± 3.8	−8.0 ± 16.8	NS
Protein	−14.5 ± 91.4	−12.9 ± 92.7	3.4 ± 12.3	NS
GH6.2	$GH6.2^a/GH6.2^a$	$GH6.2^a/GH6.2^c$	$GH6.2^c/GH6.2^c$	
	(n = 95)	(n = 31)	(n = 2)	
Milk	−324.5 ± 56.1	−595.4 ± 80.4	−1281.8 ± 308.4	0.0004
Fat	−11.1 ± 2.0	−19.8 ± 2.8	−23.0 ± 10.9	0.0217
Protein	−11.5 ± 1.4	−19.2 ± 2.1	−22.6 ± 7.9	0.0037

Values are means ± SE expressed in kg.

morphisms are presented in Table 4. Bulls with $GH4.1^c/GH4.1^c$ genotype had higher milk yield compared to $GH4.1^c/GH4.1^t$ ($P \leq 0.005$) and $GH4.1^t/GH4.1^t$ ($P \leq 0.0022$) bulls, respectively. Bulls with $GH4.1^c/GH4.1^c$ genotype had higher kg fat ($P \leq 0.0076$) and kg protein ($P \leq 0.0018$) compared with bulls with $GH4.1^c/GH4.1^t$ genotype. Similar effects were observed with the GH6.2 polymorphism on milk production traits with the $GH6.2^a$ allele being the favorable allele (Table 4). No associations ($P \geq 0.05$) were found between the genotypes of the other GH polymorphisms (GH1, GH4.2, GH5 and GH6.1) and milk production traits. The average effects of alleles of GH4.1 and GH6.2 polymorphisms are given in Table 5. The average effects of the gene substitution for GH4.1 and GH6.2 were similar and amounted to ± 300kg for milk yield, ±8 kg for fat content and ±7 kg for protein content per lactation.

DISCUSSION

DNA sequence variations have been conventionally identified as restriction fragment length polymorphism (RFLP). However, RFLP can only be detected when DNA polymorphisms are present in the recognition sequences for the corresponding restriction enzymes or when a deletion/insertion is present in the region detected by a specified probe. SSCP analysis overcomes this limitation and allows the detection of any sequence changes which lead to mobility differences of single-

TABLE 5

Average additive gene substitution effects of alleles of GH4.1 and GH6.2 polymorphisms on milk yield, fat content and protein content of milk

	Traits		
GH alleles	Milk (kg)	Fat (kg)	Protein (kg)
$GH4.1^c$	43.0	1.1	1.0
$GH4.1^t$	−253.6	−6.5	−5.9
$GH6.2^a$	44.9	1.1	1.0
$GH6.2^c$	−282.9	−6.8	−6.1

stranded DNA molecules. Using this technique, we detected a total of six polymorphic sites in the GH gene, indicating that SSCP is a useful tool to identify DNA polymorphisms.

The 5′ region of the GH gene contains regulatory sequences which control the expression of GH and interact with a large number of *cis*-acting (CRONE *et al.* 1990) and *trans*-acting factors (COURTOIS *et al.* 1990). Modulation of the affinity of binding of any of these factors by minor sequence changes in the region may affect GH transcription and thus the concentration of GH measured in the blood. Therefore, the GH1 SSCP involving a deletion/insertion of a TGC repeat in the 5′ flanking region (506–524 bp upstream from the transcription initiation site) of the gene becomes of interest for further study. However, no significant associations between the GH1 polymorphism and milk production traits were observed, suggesting that the repetitive TGC sequence may not be involved in the regulation of the gene.

Using Southern hybridization with bovine GH cDNA as a probe, COWAN *et al.* (1989) and HILBERT *et al.* (1989) detected a *Msp*I-RFLP in the GH gene and mapped it to the third intron. Restriction fragment analysis of the GH4 fragment that covers the third intron confirmed the presence of this polymorphic site. Our analysis revealed that the 345-bp fragment (GH4) habors a nonpolymorphic *Msp*I site (position 1438–1439) which cleaves into two fragments of 59 and 286 bp upon digestion with *Msp*I. The 286-bp fragment is further cleaved to yield a 109- and a 177-bp fragments when the polymorphic *Msp*I site is present. Using the sequence information of GORDON *et al.* (1983), we mapped the polymorphic *Msp*I site to position 1547–1548. Sequence analysis of the GH4 fragment showed that this polymorphism was caused by a C to T transition at position 1547. Our sequence did not agree with that of HØJ *et al.* (1993) who reported that the loss of the *Msp*I site resulted from the insertion of a T at position +837 (relative to translation start codon) and a C-to-G change at position +838. However, the sequences flanking the *Msp*I site in our analysis agreed with the GH gene sequence reported by WOYCHIK *et al.* (1982) but did not match the sequence reported by GORDON *et al.* (1983) where a T between 1540–1541 and a G between 1549–1550 were absent. *Msp*I-RFLP analysis of all the samples showed that the A_4 and C_4 SSCP alleles were all *Msp*I (+), while the B_4 allele was *Msp*I (−), showing the consistency of the two methods.

Estimates of genotypic effects of the GH4.1 variants in Table 4 indicate an association with milk production traits. In our study of the GH4.1 polymorphism, the $GH4.1^c$ allele (*Msp*I+) was the favorable allele with a substition effect of 300 kg for milk yield, 8 kg for fat content, and 7 kg for protein per lactation. Our results contradict the studies of HØJ *et al.* (1993), who reported a positive association of of *Msp*I(−) with milk fat content in Red Danish and Norwegian dairy cattle, and LEE *et al.* (1994), who also observed a positive association of *Msp*I(−) with milk fat content in Holstein cows. Based on a 9 fragment-allelic combination, LAGZIEL *et al.* (1996) recently reported a GH haplotype of *Bos indicus* origin that was positively associated with milk protein percentage.

The GH6.1 polymorphism is also known as *Alu*I-RFLP, which was previously characterized by ZHANG *et al.* (1992, 1993) and LUCY *et al.* (1993). It is caused by a C-to-G nucleotide change in the fifth exon of the GH gene, which gives rise to two alleles that are responsible for alternative forms of bovine GH with a leucine or valine amino acid residue at position 127. Restriction analysis of all 128 bulls revealed that the *Alu*I restriction recognition site was present in the B_6 and C_6 (*Alu*I+) but not in the A_6 allele (*Alu*I−). EPPARD *et al.* (1992) demonstrated that lactating Holstein cows injected with valine variant recombinant-derived bovine GH, had greater milk yield than cows that received leucine variant GH. However, LEE *et al.* (1993) and LUCY *et al.* (1993) reported a decreased milk yield associated with valine variant bovine GH in Holstein cows. Further studies by SCHLEE *et al.* (1994) in German Black and White bulls revealed that animals homozygous for the leucine variant had higher plasma levels of GH than their heterozygous counterparts. In our sample, the frequency of the $GH6.1^g$ allele (equivalent to the valine variant) was low and only one $GH6.1^g/GH6.1^g$ homozygote carrier could be identified. Differences of least square means between the homozygote $GH6.1^c/GH6.1^c$ (n = 106) and the heterozygote $GH6.1^c/GH6.1^g$ (n = 21) for milk yield, fat content and protein yield were not significant ($P \geq 0.05$).

The GH6.2 polymorphism is due to an A-to-C transversion which changes the codon AGG to CGG in the fifth exon of the gene. However, either triplet codes for the same amino acid, arginine. This mutation changes the recognition sequence (C↓TNAG) of the restriction enzyme *Dde*I. Therefore, this polymorphism could also be identified as *Dde*I-RFLP. Assessment of the 128 individuals investigated indicated that all 95 individuals homozygous for $GH4.1^c$ were also homozygous for $GH6.2^a$, all 29 heterozygotes $GH4.1^c/GH4.1^t$ were heterozygotes $GH6.2^a/GH6.2^c$, and of the four homozygous $GH4.1^t/GH4.1^t$ individuals, two were $GH6.2^c/GH6.2^c$ homozygotes and two were $GH6.2^a/GH6.2^c$ heterozygotes. This observation indicates extensive linkage disequibrium between the two polymorphisms and may explain why significant associations were found for both polymorphisms. The effects of GH4.1 and GH6.2 on milk production traits could be attributed to variations in the expression of the bovine GH or due to tight linkage disequilibruim with another mutation.

Previous studies using Southern analysis revealed RFLPs in the bovine GH gene with *Bgl*I, *BamH*I, *EcoR*I,

*Pst*I, *Pvu*II and *Taq*I, all of which were caused by an insertion/deletion event of a 1-kb DNA fragment in the 3′ region of the gene (HALLERMAN *et al.* 1987; COWAN *et al.* 1989; HILBERT *et al.* 1989). The exact location of the deletion/insertion is unknown but was proposed to involve a segment 300 to 1300 bp downstream from the polyadenylation site (HILBERT *et al.* 1989). Analysis of the PCR products of the GH7 fragment (454 bp) that covers the polyadenylation site and 429 bp 3′ from the site revealed that in some individuals, a single major band of 454 bp (expected) was present, while in others, beside the same major band with approximately half of the intensity, two additional minor bands of 500 and 550 bp, respectively, were present (data not shown). Assessment of 108 bulls revealed that those individuals with a single major band were all homozygous for *Msp*I (+), while the ones showing one major band with less intensity plus two minor bands were all *Msp*I (+/−) animals with few exceptions. Based on the observation by HØJ *et al.* (1993) that the insertion allele was linked to the *Msp*I (+) allele and the deletion allele was linked to the *Msp*I (−) allele, we therefore conclude that those animals with a single amplification are homozygous for the insertion of the 1-kb fragment, and those with two additional bands are heterozygous for the deletion and insertion. We further conclude that the GH sequence reported by (GORDON *et al.* 1983) contains part of the 1-kb insertion at the 3′ end as hypothesized by (HALLERMAN *et al.* 1987) and that the P14 primer (reverse primer for GH7, position 2829–2850) is located in this region. The presence of the two minor bands in heterozygous animals could be explained as nonspecific amplifications due to the elimination of the P14 primer binding site by deletion of the 1-kb fragment.

Associations between single genes or putative QTL and quantitative traits without taking into account the breeding structure and gene flow may be spurious because of non random association of gametes (KENNEDY *et al.* 1992). In this study, the estimated breeding values are based on an animal model that accounts for the relationships between all animals and the bias of selection. The birth year of the bulls was fitted to account for genetic trend in improvements during the past 40 years. However, a definitive conclusion requires segregation studies.

In summary, our study revealed six different polymorphisms in the bovine GH gene, four of which have not been reported previously. The association of two of these polymorphisms GH4.1 and GH6.2 with milk production traits suggests that these markers may be useful for selection at the DNA level.

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