We studied sequence variations in the regulatory region of the bovine growth hormone receptor gene. A polymerase chain reaction (PCR)-based method for detecting Alu I, Acc I, and Stu I restriction fragment length polymorphisms (RFLPs) in the 5' flanking region of the bovine growth hormone receptor gene was developed and tested for association with milk-related traits in Holstein bulls. Allele frequencies of the polymorphisms in two groups of Holstein progeny-tested bulls born from 1950 to 1970 and in the 1980s, respectively, were estimated. The allele frequency of the Alu I(−) allele was 0.63 and 0.42 in the bulls from 1950 to 1970 and in the 1980s, respectively. The frequency of the Stu I(−) allele was 0.14 and 0.07 in the two respective bull groups. Allele frequency for Acc I(−) allele was about 0.22 in both bull groups. The differences in allele frequencies for the Alu I polymorphism in the two bull groups were significantly different (P < .005). The Alu I(+/+) bulls had a higher estimated breeding value (EBV) for fat (P < .016) than Alu I(−/−) bulls. The average effect of allele substitution for the Alu I polymorphism was $8 for fat EBV. The Alu I polymorphism could be further evaluated for use in marker-assisted selection in dairy cattle.

A main goal of animal breeders is to select superior animals for breeding. Screening favorable alleles for selection at the DNA level provides an ideal tool for marker-assisted selection. In dairy cattle, promising candidate genes for many traits are in the growth hormone (GH) axis. Growth hormone is a polypeptide hormone with diverse biological activities including somatogenic (growth promoting), lactogenic, insulin-like, and diabetogenic effects. In dairy cows, exogenous administration of GH stimulates growth and milk production (Peel and Bauman 1987). Similarly, sequence variations in the GH gene and their effects on milk-related traits are well documented (Ho et al. 1993; Lagziel et al. 1996; Yao et al. 1996). However, the mechanism by which GH acts in the cow to effect these physiological changes is not fully understood.

Pertinent to the development of a genetic model for the GH axis is the role of GH receptor (GHR). In murine species, GHR mediates mammary development, and altered GH and/or GHR affected mammary growth and differentiation (Feldman et al. 1993). Growth hormone binds to proteins found in the cell cytoplasm, blood plasma, and on cell surfaces, and the binding of GH to the membrane-bound receptor is the first step in the biological action of GH. Following a ligand-induced dimerization of the GHR, multiple cascades are initiated in GH signaling. GH and/or GHR mutants that cannot induce dimerization are therefore biologically inactive (Cunningham et al. 1991). Mutations in the GHR gene have been associated with Laron-type dwarfishm in humans (Godowski et al. 1989) and sex-linked dwarfism in chickens (Burnside et al. 1992). This suggests that variations in the regulatory or structural sequence of GHR gene would be of interest because of its role in mammary development. Falaki et al. (1996) reported an association of bovine GHR TaqI restriction fragment length polymorphism (RFLP) with milk protein percentage in Italian Holsteins. The 5' regulatory region contains constitutive promoter elements, enhancers, repressors, the determinant of tissue-specific gene expression, and other responsive elements (Cooper 1992), therefore sequence variations in the regulatory region may be of importance. Our objective was to study the sequence variations in the regulatory region of the bovine GHR gene and their association with milk-related traits in Holsteins.

Materials and Methods

DNA Samples

Semen samples were randomly collected from 301 progeny-tested Holstein bulls.
born from 1950 to 1992. They were obtained from Centre d’Insemination Artificielle du Quebec (Saint Hyacinthe, Quebec, Canada), Western Ontario Breeders Inc. (Woodstock, Ontario, Canada), and United Breeders Inc. (Guelph, Ontario, Canada). Genomic DNA from semen was isolated as previously described (Zadworny and Kuhnlein 1990).

DNA Amplification with Polymerase Chain Reaction and Sequence Analysis

Based on the published nucleotide sequence of the promoter region and exon 1 of the bovine GHR gene (Heap et al. 1995), two pairs of oligonucleotide primers were synthesized to amplify two fragments. One fragment was 836 bp in length (position -1871 to -1036) containing a polymorphic AluI site. The other fragment was 1119 bp in length (position -1056 to +63) which harbored AccI and StuI sites. The sequence of the forward and reverse primers, respectively, were 5’-TGCGTGCAACAGACCTCAACC-3’ and 5’-AGCAACCCCACGTCTGGCAT-3’ for the 836 bp fragment, and 5’-ATGCCAGCTGGGTTGCT-3’ and 5’-GGCAACAGCTGGGGTTGGA-3’ for the 1119 bp fragment. PCR for the 836 bp fragment was performed in a reaction volume of 25 µl using 100 ng of DNA, 0.25 µM of each primer, 1× PCR buffer (50 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl pH 9.0), 5% deionized formamide, 160 µM dNTP, and 0.625 units of Thermus thermophilus (Tth) DNA polymerase (Pharmacia, Baie D’Urfé, Quebec, Canada). Similar conditions were used for the 1119 bp fragment except that no deionized formamide was included. Amplification was carried out for 35 cycles at 92°C for 60 s, 66°C for 80 s, and 72°C for 120 s after preheating at 94°C for 10 min using a DNA thermal cycler (Perkin Elmer Cetus Corp.). For RFLP analysis, 10 µl of the 836 bp fragment products were digested with 5 units of AluI (Pharmacia), and two 10 µl aliquots of the PCR product from the 1119 bp fragment were digested with 5 units of AccI or StuI (Pharmacia). They were all digested at 37°C for at least 2 h. The digested DNA fragments were then separated by electrophoresis in 2% agarose gel in 1× TPE (90 mM Tris-phosphate, 2 mM EDTA). The gel was stained with ethidium bromide and visualized under UV light. A total of 301 bulls were genotyped. Amplified DNA fragments were cloned into pUC18 plasmid for sequence analysis. Sequences were determined by the dideoxy-chain termination method of Sanger et al. (1977) using a T7 sequencing kit (Pharmacia) with [35S]dATP as the labeled nucleotide.

Statistical Analysis

Of the 301 samples, 2 subsamples were made representing bulls progeny tested during 1950–1970 (n = 67) and the 1980s (n = 61), respectively. Differences in allele frequencies of the AluI, AccI, and StuI polymorphisms in the two groups of Holstein bulls were tested by chi-square test. The estimated breeding values (EBVs) of the bulls for milk, fat, and protein yields were obtained from the February 1997 Canadian National Dairy Evaluations, which were based on the best linear unbiased procedure (BLUP) with an animal model. The model included fixed effects of herd-year-season and age group of sires. The effect of GHR genotypes on the EBVs for milk, fat, and protein yields were then analyzed using least squares methods. The estimated breeding values from the national evaluation system represent the best available estimates of the additive genotype of the bulls. The effect of birth year of the bulls was included in the model to account for genetic trend. The model used was as follows:

\[ Y_{ijklm} = \mu + Year + AluI_j + AccI_k + StuI_l + e_{ijklm} \]

where \( Y_{ijklm} \) is the breeding value (milk, fat, or protein yields) of the \( m^{th} \) bull; \( \mu \) is the least squares estimate of the mean of the trait; \( Year\) is the effect of the \( i^{th} \) birth year of the bull (genetic trend); \( AluI_j \) is the effect of the \( j^{th} \) AluI genotype (\( j = 1, 2, 3 \)); \( AccI_k \) is the effect of the \( k^{th} \) AccI genotype (\( k = 1, 2, 3 \)); \( StuI_l \) is the effect of the \( l^{th} \) StuI genotype (\( l = 1, 2 \)); and \( e_{ijklm} \) is the random residual effect. Type III sum of squares were used to evaluate the effect of GHR polymorphisms. Differences in the estimated breeding values were compared by least squares procedures. Average additive gene substitution effects of the milk-related traits were calculated for the alleles of the GHR polymorphisms, which showed significant effects on the trait according to Falconer and Mckay (1996). All the 301 bulls types were used in the association analysis.

Results

Three RFLPs in the 5’ flanking region of the bovine GHR gene were detected. There were three AluI sites in the 836 bp fragment. The digested AluI(-/-) PCR product exhibited three fragments of 747 bp, 75 bp, and 14 bp (not detected on the gel). For the AluI(+/+) PCR product, the 747 bp fragment was cleaved into two fragments of 602 and 145 bp. Figure 1A shows the restriction patterns of the three genotypes—AluI(+/+), AluI(+/−) and AluI(−/−)—upon digestion of the PCR products. Sequence analysis of the polymorphic AluI site revealed a mutation at position −1182. The mutation was an A-to-T transition. The number of individuals with different genotypes and allele frequencies of the three polymorphisms in the 5’ flanking region of the bovine GHR gene in two groups of Holstein bulls are presented in Table 1. The frequency of the AluI(−) allele was 0.63 and 0.42, and that of AluI(+) was 0.37 and 0.58 in bulls of 1950–1970 and 1980s, respectively. The allele frequencies between the two groups were significantly different ($\chi^2_{df=1} = 8.84; P \leq 0.005$).

The 1119 bp fragment contained only single restriction sites of AccI and StuI. Upon digestion with AccI, the AccI(+/+) PCR product was cleaved into two fragments of 958 bp and 161 bp (Figure 1B). Sequence analysis of the AccI site revealed a mutation at position −892, and the mutation was a C-to-T transition. The frequency of the AccI(−) allele was about 0.22 in the two groups of bulls. When the 1119 bp fragment was digested with StuI, the StuI(+/+) was restricted into 824 bp and 295 bp fragments. No StuI(−/) genotype was detected in the samples screened (Figure 1C). The StuI polymorphism was caused by a C-to-T transition mutation at position −232. The frequency of the StuI(−) allele was 0.14 and 0.07, and that of the StuI(+) allele was 0.86 and 0.93 in the bulls of the 1950–1970 and 1980s, respectively. The allele frequency between the two groups was not significantly different ($P \approx 0.05$).

The effects of the three GHR polymorphic sites on the breeding values for milk, fat, and protein yields were examined in 301 Holstein bulls using least squares methods. Allele frequencies for AluI(+), AccI(+), and StuI(+) alleles in the 301 bulls genotyped were 0.53, 0.77, and 0.94, respectively. Least square means of the GHR genotypes on the EBVs for milk, fat, and protein EBV ($P \approx 0.05$) compared with the AluI(−/−) and AluI(+/−) bulls. Bulls with StuI(+/+) genotype had higher fat content ($P \approx 0.016$) compared to AluI(−/−) and AluI(+/−) bulls. Bulls with StuI(+/+) genotype had higher fat EBV ($P \approx 0.008$) and protein EBV ($P \approx 0.011$) compared with bulls with StuI(−/−) genotype. There was no significant association ($P \approx 0.05$) between AccI genotypes and milk-related
traits. The average effect of the Alu(−) allele was 42.54, −3.96, and −1.35 for milk, fat, and protein EBVs, respectively, and that for the Alu(+) allele was −38.8, 3.55, and 1.12 for milk, fat, and protein EBVs, respectively. The average effects of the allele substitution for Alu amounted to about ±8 for fat EBV.

Discussion

The approach for testing candidate alleles looks for a relationship between the phenotype and a genetic variant that may or may not alter gene structure and the function or expression of the gene product. This type of study provides biological information to augment statistical insight into the nature of genes affecting quantitative variation. Comparison of allele frequencies between bulls born between 1950 and 1970 and bulls of the 1980s indicated that the frequency of the Alu(+) allele had increased and consequently the Alu(−) allele had decreased. Genetic improvements in dairy cattle during the time interval of the two bull populations could be associated with the changes in the observed frequencies of the Alu haplotypes. However, genetic drift could not be excluded. Estimates of the effects of the GHR-Alu variants in Table 2 indicate a significant difference in the fat EBV between the Alu(+/+) and Alu(−/−) genotypes. Alu(+) was the favorable allele, which was consistent with the allele frequency data presented in Table 1. The substitution effect of the Alu(+) allele was about 8 for fat EBV.

The Stul(+) allele is almost fixed in the population screened, however, it was associated with fat and protein EBVs. Even though the allele substitution effect of the Stul polymorphism was not calculated because of a lack of Stul(−/−) individuals, the results from the allele frequency changes (Table 1) and the association analysis (Table 2) combined to suggest that the Stul(+) allele was favorable. Stul(−/−) individuals have not been detected in other cattle breeds (Ayrshire, Boran, and N’Dama) (Aggrey SE, unpublished data). It may also suggest that the Stul(−) allele is either very rare or deleterious. Falaki et al. (1996) reported an association of GHR-TaqI polymorphisms with milk protein content. However, their study used Southern blotting and the TaqI polymorphisms were unresolved and unmapped, which led to low numbers of bulls in some groups, thereby leading to a limited statistical inference. The GHR-Alu and GHR-Stul polymorphisms appear to be associated with protein and fat yields, however, the causative genes are not yet known. It is possible that these markers are linked to a causative gene on chromosome 20 or are in linkage disequilibrium with a causative site at some other genes. If there are linked effects, information about the phase and strength of the linkages would be required prior to their utilization in any form. Georges et al. (1995) reported among others a milk-related QTL on chromosome 20 segregating in

Table 1. The genotypes and allele frequencies of Alu, Acc1, and Stul polymorphisms in the 5’ flanking region of the bovine growth hormone receptor (GHR) gene in two groups of Holstein bulls

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Groupa</th>
<th>Genotypes</th>
<th>Allele frequency</th>
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<tr>
<td></td>
<td></td>
<td>−/−</td>
<td>+/−</td>
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<tr>
<td>Alu</td>
<td>1</td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11</td>
<td>29</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>Acc1</td>
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<td>0</td>
<td>19</td>
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<td></td>
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<td>8</td>
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<td>0</td>
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the U.S. Holstein population. We do not know whether the same QTL is segregating in the Canadian Holstein population and consequently its potential effect on the results presented in this study could not be excluded.

Estimated association between markers in single genes or putative QTL and quantitative traits without taking into account the breeding structure, and gene flow may be spurious because of nonrandom association of gametes (Kennedy et al. 1992). Testing of the 5′-flanking GHR polymorphism effects using a mixed model with an animal model analysis may remove the possible nonrandom associations. However, analysis based on actual records of daughters of the genotyped bulls could mean that the number in each subclass of observations of fixed factors would be small. In addition the data are not well connected, and thus the fixed factors would have very poor estimates (Sabour et al. 1996). In this study the estimated breeding values are based on a mixed model procedure with an animal model that accounts for the relationship between all animals and the bias of selection. The birth year of the bulls was fitted to account for the genetic trend in improvement in the bull population used. Furthermore, the repeatability of the breeding values of the bulls was high (95–99%), and most bulls involved in this study were not related; however, family segregation study is required for a definitive conclusion.

Differences in fat EBV between AluI genotypes could have potential use in manipulating milk composition. This marker could be further evaluated for marker-assisted selection and the developed PCR methodology would expedite screening of large numbers of animals required for such studies.

References

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