RELATIONSHIPS OF POLYMORPHISMS IN THE BOVINE LEPTIN GENE WITH DIFFERENCES IN CARCASS TRAITS OF BEEF CATTLE

HONORS RESEARCH PROJECT

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Introduction

Leptin is a 16-kilodalton protein produced by the obesity (*ob*) gene. It was first discovered in mice by Jeffrey Friedmann's research team at Rockefeller University in New York City in 1994 (Gura, p.751). Defects in both the mutant and wild-type copies caused the animals to act as if in starvation, depositing up to three times the body fat as mice without defective copies. Leptin was traced back to fat cells, called adipocytes, where it is produced and then released into the bloodstream. In addition, leptin receptor locations have been identified in the brain, also suggesting that leptin plays a role in appetite regulation and weight control. Specifically, leptin receptors have been expressed in four main regions of the hypothalmus, including two that have been previously noted for involvement in feeding and metabolism (the articulate nucleus and the paraventricular nucleus) (Gura, p.751). Although it has been determined that leptin is related to fat deposition, its specific role is not yet known.

Even though the specific cause of fat deposition in animals is not yet known, the involvement of leptin in this process is thought to be very important, in both humans and animals. In the bovine species, the leptin gene has been mapped to chromosome four (Wilkins and Davey, p.376). The availability of highly polymorphic markers within and adjacent to the bovine leptin gene will facilitate genetic studies in cattle to determine the specific roles leptin plays (Wilkins and Davey, p.376). A study by Wilkins and Davey (1997) revealed a polymorphic microsatellite in the bovine leptin gene, this being microsatellite 5' AAAAAAAAAAAAAAAATATATATATATATATATATA 3' in the 5' UTR region of the gene. This fragment corresponds to nucleotides 247 to 282 in the deposited sequence (GenBank Accession No. U50365). The microsatellite studied proved to be highly polymorphic with 18 alleles revealed after genotyping 97 individuals (Wilkins and Davey, p.376).

Additionally, another microsatellite and several point mutations have also been

detected in the bovine leptin gene. A microsatellite of the composition 5' GATA $(CA)_n$ CTAG 3' has been detected in the DNA flanking the 3' end of the obesity gene (Stone et al., 1996 [GenBank Accession No. G18586]). This microsatellite has at least four alleles (Fitzsimmons et al., 1998). The two most polymorphic point mutations are readily detected by restriction enzyme digestion of segments amplified by the polymerase chain reaction (PCR) (Lien et al., 1997). Polymorphisms such as these are referred to as PCR-amplified restriction fragment length polymorphisms (PCR-RFLPs).

Problem Identification and Justification

The ob gene, which produces the protein leptin, is believed to be involved in the regulation and deposition of fat tissue. Therefore, it may play a significant role in genetic studies of cattle. In this project, the microsatellites described by Wilkins and Davey (1997) and Stone et al. (1996 [GenBank Accession No. G18586]), together with the PCR-RFLPs characterized by Lien et al. (1997), were used to identify polymorphisms in the bovine leptin genes of 139 bulls in The Ohio State University Angus beef cattle research herd, located at the Eastern Ohio Resource Development Center (EORDC) in Belle Valley. Extensive research is ongoing in this herd concerning selection for blood serum insulin-like growth factor I (IGF-I) concentrations (Davis et al., 1995; Davis and Simmen, 1997). The DNA collected for that study were analyzed in this study.

Bulls were used in this study so that relationships with both growth and carcass traits could be analyzed. Such data were already collected for the IGF-I concentration studies by Davis et al.(1995). The carcass traits include backfat thickness, ribeye area, kidney, pelvic and heart fat percentage, hot carcass weight, cutability marbling and yield grade.

Justification for finding and using genetic markers in the bovine leptin gene and other fat/growth associated gene(s) lies in the economic importance of carcass traits desired for beef cattle. Public demands for leaner, higher quality meat govern the direction of the

beef industry. Currently, meat quality is only regulated by producers through feed intake and selection programs. However, if a polymorphic genetic marker for the leptin gene is identified and the role leptin plays in fat deposition is determined, then research on how to genetically control fat content of meat would progress greatly. Cattle with leaner body composition could then be selected via markers at the leptin locus, resulting in carcasses with either a higher or lower marbling score and a higher quality grade. This research also has the potential to fulfill a variety of consumer desires (i.e. niche markets).

Identification of a genetic marker for the leptin gene should also reduce costs to the producer. Since the energetic cost of producing muscle is less than that of producing fat, it is anticipated that leaner animals could be raised with lower feed costs.

Objectives

In this project involving the study of polymorphisms and their relation to growth and carcass traits, three objectives were identified. The first was to determine if the Angus bulls at EORDC were polymorphic for three segments of the bovine leptin gene. This needed to be determined first so that alleles and genotypes could be identified for each of the animals for each of four markers.

The second objective was to evaluate the allelic frequencies in the line selected for high IGF-I concentrations and in the line selected for low IGF-I concentrations and to test for significant differences. Animals selected for high IGF-I concentrations have lower weights and less gain, while those selected for low IGF-I concentrations have higher weights and more gain (Davis et al., 1995). Significant differences between these two lines in allele frequencies of the leptin gene could point to differences in fat content of the animals.

The third objective was to evaluate the relationship of detected marker variation with differences in the carcass traits of these Angus bulls. This was the ultimate goal of this research, and any relationship found could aid in determining how leptin regulates fat

deposition in the bovine species.

Procedures

In this study, DNA previously collected on 139 Angus bulls from the EORDC beef cattle research herd in Belle Valley, Ohio, was analyzed to determine polymorphisms in the leptin genes of these cattle. This DNA was on storage in the Animal Genetics Lab in the Department of Animal Sciences at The Ohio State University. Bulls were chosen from both the line selected for high IGF-I concentration and the line selected for low IGF-I concentration. The bulls used were born in the following seasons: Fall 1995, Spring 1996, Fall 1996 and Spring 1997.

The first step was to perform a polymerase chain reaction (PCR) amplification on each sample of DNA from these bulls. The components of this reaction were as follows: 100 ng/μl template DNA, 10X PCR standard reaction buffer, 125 m dNTPs, 1 μl each specific primer, 10 pmoles each primer ETH121 and 1.0 μl Taq polymerase. Primers used for each marker included:

Wilkins & Davey Microsatellite (WD-MS):
Forward: 5' TTGTAATCCTGCAATATCTTGTCC 3'
Reverse: 5' TAAACAGGCCGTAGCAGTACAG 3'

Stone Microsatellite (STONE-MS):
Forward: 5' GATGCAGCAGACCAAGTGG 3'
Reverse: 5' CCCATTGCTAGAACCCAGG 3'

PCR-RFLP:
Forward: 5' GGCTGGACGCAAAGGGCAGAGT 3'
Reverse: 5' CCCTGACGCCGCATTTCCCTA 3'

All primer sets were amplified in separate reactions. The PCRs were performed using a Perkin-Elmer thermocycler. The samples were subjected to 31 amplification cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds and

extension at 72 C° for 1 minute. The final extension step was lengthened to 7 minutes to ensure that polymerization was complete. The samples were run on an agarose gel to confirm amplification.

The PCR products amplified with the PCR-RFLP primers were then digested using Hinf1 and BsaA1 restriction endonucleases. The digestion included 4 μl PCR sample, 1μl enzyme, 3 μl specific buffer and 4.6 μl water. The samples were incubated for 2 hours at 37 °C, and then the reaction was stopped by adding 4 μl of loading buffer.

After PCR amplification and digestion were completed, products from these reactions were transportation to Dr. Christopher Weghorst's genotyping laboratory in The James Cancer Hospital at The Ohio State University. The amplified products from each sample were combined as follows: 9 μl WD-MS, 4 μl Stone-MS and 9 μl PCR- RFLP. Then 6 μl of this combination was added to 4 μl formamide and 0.5 μl ROX internal lane standard (GS2500 Applied Biosystems). The samples were heat-denatured at 95 °C for 4 minutes, chilled on ice and loaded onto a standard 6% polyacrylamide denaturing sequencing gel in an ABI 377 DNA Sequencer of Applied Biosystems. The PCR products were dye-labeled using the fluorescent dyes fam (blue) for WD-MS, tet (green) for Stone-MS and hex (yellow) for PCR-RFLPs. The PCR products were automatically sized by reference to the internal lane standard, and genotypes were determined with the aid of the Perkin-Elmer/ABI Genotyper software present in the Animal Genetics Lab.

Once genotypes were determined, allele frequencies could be found. Allelic frequencies for each selection line were calculated simply by dividing the counts for each allele in each selection line by the total count for all alleles found in that line. Total allele frequencies were also calculated by dividing the number of a specific allele by the total

number of alleles in each group (WD-MS, Stone-MS or PCR-RFLP). Significant difference in allelic frequencies between selection lines were determined using a Chi-square analysis. A level of probability of .05 considered indicative of significance.

Next, the genotypes were encoded numerically and entered into the SAS computer system to be analyzed with carcass data already collected on these bulls by Davis et al. (1995) for studies using the General Linear Models procedure. The statistical model included fixed effects of genotype, birth year, season of birth, IGF-I selection lines, and age of dam. A covariate for on-test age of calf was also included in the model.

Results

Two data analyses were performed in this experiment. The first was a Chi-Square analysis of the allele frequencies at each typed variable site within the leptin gene. The second was an analysis of the relationship between genotype and carcass traits, which was performed using the General Linear Models procedure of the SAS computer program.

Allelic frequencies were calculated for each mutation site, both within the high and low IGF-I lines and across both lines. Refer to Table 1 for an enumeration of allele frequencies. At the WD-MS mutation site, six alleles were found in 137 Angus bulls, these being detected as amplified segments of 177, 184, 186, 190, 192 and 209 base pairs in length. Chi-square analysis of the frequencies of these alleles indicated significant differences between the high and low IGF-I selection lines ($P<.01$).

For the Stone microsatellite, 131 bulls were analyzed. Two alleles were found, and these were detected as amplified segments of 135 and 144 base pairs in length. It is important to note that neither of these alleles have been previously reported. Chi-square analysis detected significant differences in allele

frequencies between the high and low IGF-I selection lines (P<.05).

At the PCR-RFLP mutation site, two alleles were found in 139 bulls. The B allele was associated with an uncut fragment length of 170 base pairs, while the C allele was represented by two cut fragments, lengths of 81 and 89 base pairs. Chi-square analysis of the frequencies of these alleles in the high and low IGF-I selection lines showed no significant difference between the lines (P=.55).

Refer to Table 2 for relationships between genotypes and carcass traits. Analysis of variance showed that the WD microsatellite had a significant effect (P<.02) on ribeye area. Also, it is important to note those traits for which P<.20 because of the trends that they may reveal. These traits were marbling score for both the Stone microsatellite and the PCR-RFLP (P=.15 and P=.19, respectively) and KPH for the PCR-RFLP (P=.20). No other significant relationships were found.

Five previously unreported alleles were found during the course of this experiment. At the WD-MS site, new alleles consisting of 190, 192 and 209 base pairs were found, and at the Stone-MS site, new alleles consisting of 135 and 144 base pairs were found. It is important to note that the only two alleles found at the Stone-MS locus in these Angus bulls were these two alleles.

Discussion

It was surprising to find only one significant relationship between genotype and carcass traits, because leptin has been shown to be involved in fat deposition (Gura, p.751). This lack of significance may be due to several causes.

First, the animals studied were all purebred Angus bulls, and the sample therefore lacked the genetic variaton that would be expected of a group of crossbred animals or of a diverse group of breeds.

In the experiment by Wilkins and Davey (1997) in which the polymorphic WD-MS locus was discovered, the sample consisted of different breeds of dairy cattle. Also, in another experiment by Pomp et al. (1997), the sample consisted of Limousin, Holstein, Hereford, Angus, Brahman, and Brangus breeds. This sample was used to find a PCR-RFLP with *Sau*3AI restriction endonuclease (Pomp et al., 1997). It is recommended that future studies of the leptin gene include diverse breeds, thereby increasing the genetic variation within the sample.

Secondly, genotypes of only 139 Angus bulls were analyzed The sample size was small due to the limited number of DNA samples available from bulls that had already been slaughtered and from which carcass data had been obtained and recorded. In future studies, a larger sample size may reveal more significant genotype-carcass trait relationships.

The final factor affecting the outcome of these results is that the exact role leptin plays in fat deposition has not yet been determined. The carcass traits examined in this experiment may have been too superficial to elucidate the effects of the leptin genotypes on the biology of the animals analyzed. Comparison of leptin polymorphisms and actual serum leptin concentrations may be more revealing. This comparison is especially recommended because, although only one significant relationship was found between genotypes and carcass traits, two of the sites revealed significant allelic frequency differences between the high and low IGF-I selection lines. This result may be an indicator of other effects of leptin genotypes in the bovine system.

Conclusions

In this experiment, leptin genotypes and allelic frequencies were determined for 139 yearling

Angus bulls. At the WD-MS and Stone-MS sites, significant differences in allelic frequencies were found between the lines selected for high vs low blood serum IGF-I concentration. However, at the PCR-RFLP site, there was no significant difference in allelic frequencies between the high and low IGF-I selection lines.

Effects of leptin genotypes on carcass traits were also examined. The WD-MS genotypes had a significant effect ($P<.02$) on ribeye area. Both the Stone microsatellite and the PCR-RFLP had noteworthy effects ($P<.20$) on marbling score, and the PCR-RFLP also had an effect on KPH ($P=.20$). No other significant effects on carcass traits were found.

Table 1. Allele Frequencies and Chi-Square Analysis of Allele Frequencies

W-D Microsatellite*:

Allele	Frequency in High Line	Frequency in Low Line	Total Allele Frequency
177	.51	.63	.57
184	.15	.20	.17
186	.05	.01	.04
190	.02	.04	.03
192	.30	.11	.17
209	.05	.00	.03

Stone Microsatellite:

Allele	Frequency in High Line	Frequency in Low Line	Total Allele Frequency
135	.49	.36	.44
144	.51	.64	.56

PCR-RFLP:

Allele	Frequency in High Line	Frequency in Low Line	Total Allele Frequency
170	.44	.47	.45
81, 89	.56	.53	.55

Chi-Square Analysis:

Locus	Observed X^2 Value	Degrees of Freedom	P Value
WD-MS**	16.37	5	P<.01
Stone-MS**	4.51	1	P<.05
RFLP	0.31	1	P=.55

*W-D for Wilkins & Davey, who first discovered the microsatellite.

**MS=microsatellite.

Table 2. Least Squares Means and Standard Errors of Carcass Traits by Genotype

W-D Microsatellite**:

	Dependent Variable						
Independent Variable	Fat, mm	Ribeye, mm2	KPH*, %	HCW*, %	Cutability, %	Marbling	Yield Grade
Genotype (n=137)	P=.69	P<.02	P=.90	P=.25	P=.74	P=.51	P=.72
177/177	8.9 ± 0.6	76.4 ± 1.3	2.2 ± 0.1	277.8 ± 5.00	51.3 ± 0.2	4.7 ± 0.1	2.4 ± 0.1
177/190 or 177/192	9.2 ± 0.8	73.8 ± 1.8	2.2 ± 0.2	264.2 ± 6.80	51.3 ± 0.3	4.9 ± 0.2	2.4 ± 0.1
177/184	9.3 ± 0.9	80.6 ± 1.9	2.2 ± 0.2	283.4 ± 7.10	51.6 ± 0.3	4.8 ± 0.2	2.2 ± 0.1
190/190 or 192/192	8.8 ± 1.0	71.9 ± 2.1	2.1 ± 0.2	261.1 ± 7.80	51.3 ± 0.4	4.7 ± 0.2	2.4 ± 0.2
184/184 or 184/192	9.3 ± 0.9	79.1 ± 2.0	2.1 ± 0.2	274.1 ± 7.50	51.7 ± 0.4	4.7 ± 0.2	2.2 ± 0.2
192/209	4.8 ± 2.5	80.6 ± 5.5	1.7 ± 0.5	282.6 ± 20.6	53.0 ± 1.0	4.9 ± 0.5	1.7 ± 0.4
209/209	7.0 ± 2.3	84.2 ± 4.9	1.7 ± 0.4	301.1 ± 18.3	52.4 ± 0.9	5.4 ± 0.5	1.9 ± 0.4
177/186	8.8 ± 1.7	79.9 ± 3.7	2.1 ± 0.3	283.2 ± 13.8	51.8 ± 0.7	4.7 ± 0.4	2.2 ± 0.3
186/186	5.6 ± 2.0	73.5 ± 4.4	1.8 ± 0.4	272.4 ± 16.4	52.1 ± 0.8	4.0 ± 0.4	2.1 ± 0.3

Stone Microsatellite:

	Dependent Variable						
Independent Variable	Fat, mm	Ribeye, mm^2	KPH*, %	HCW*, kg	Cutability, %	Marbling	Yield Grade
Genotype (n=131)	P=.68	P=.52	P=.54	P=.89	P=.31	P=.15	P=.31
135/135	7.7 ± 0.9	77.8 ± 1.9	2.0 ± 0.2	271.9 ± 7.2	52.0 ± 0.3	4.7 ± 0.2	2.1 ± 0.1
135/144	8.2 ± 0.6	76.5 ± 1.4	2.1 ± 0.1	269.6 ± 5.5	51.8 ± 0.3	4.8 ± 0.1	2.2 ± 0.1
144/144	8.4 ± 0.7	75.6 ± 1.7	2.2 ± 0.1	272.3 ± 6.3	51.5 ± 0.3	4.5 + .01	2.3 ± 0.1

PCR-RFLP:

	Dependent Variable						
Independent Variable	Fat, mm	Ribeye, mm^2	KPH*, %	HCW*, kg	Cutability, %	Marbling	Yield Grade
Genotype*** (n=139)	P=.30	P=.50	P=.20	P=.95	P=.25	P=.19	P=.25
B/B	9.2 ± 0.8	74.3 ± 1.8	2.3 ± 0.2	271.5 ± 7.0	51.1 ± 0.3	4.5 ± 0.2	2.5 ± 0.1
B/C	8.2 ± 0.6	76.6 ± 1.3	2.0 ± 0.1	272.7 ± 4.9	51.7 ± 0.2	4.8 ± 0.1	2.2 ± 0.1
C/C	9.1 ± 0.7	76.8 ± 1.5	2.2 ± 0.1	270.9 ± 5.8	51.5 ± 0.3	4.6 ± 0.1	2.3 ± 0.1

*KPH=Kidney, Pelvic & Heart Fat, HCW=Hot Carcass Weight.

**W-D for Wilkins & Davey, who first discovered the microsatellite.

***B=170 bp, C=81 & 89 bp.

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