Evaluation of single-nucleotide polymorphisms in *CAPN1* for association with meat tenderness in cattle[1,2]

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ABSTRACT: *Micromolar calcium activated neutral protease* (*CAPN1*) was evaluated as a candidate gene for a quantitative trait locus (QTL) on BTA29 affecting meat tenderness by characterization of nucleotide sequence variation in the gene. Single-nucleotide polymorphisms (SNP) were identified by sequencing all 22 exons and 19 of the 21 introns in two sires (Piedmontese × Angus located at the U.S. Meat Animal Research Center in Clay Center, NE; Jersey × Limousin located at AgResearch in New Zealand) of independent resource populations previously shown to be segregating meat tenderness QTL on BTA29. The majority of the 38 SNP were found in introns or were synonymous substitutions in the coding regions, with two exceptions. Exons 14 and 9 contained SNP that were predicted to alter the protein sequence by the substitution of isoleucine for valine in Domain III of the protein, and alanine for glycine in Domain II of the protein. The resource populations were genotyped for these two SNP in addition to six intronic polymorphisms and two silent substitutions. Analysis of genotypes and shear force values in both populations revealed a difference between paternal *CAPN1* alleles in which the allele encoding isoleucine at position 530 and glycine at position 316 associated with decreased meat tenderness (increased shear force values) relative to the allele encoding valine at position 530 and alanine at position 316 ($P < 0.05$). The association of maternal alleles with meat tenderness phenotypes is consistent with the hypothesis of *CAPN1* as the gene underlying the QTL effect in two independent resource populations and presents the possibility of using these markers for selective breeding to reduce the numbers of animals with unfavorable meat tenderness traits.

Key Words: Calpain, Genetic Polymorphism, Meat, Quantitative Trait Loci

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Introduction

Variation in meat tenderness has significant impact on consumer satisfaction with beef, however, classical selection techniques have not been effective in eliminating the small fraction of animals yielding undesirable palatability traits. Establishing the genetic basis for variation in meat tenderness would likely aid in the development of selection criteria for improving meat tenderness in cattle.

The *micromolar calcium-activated neutral protease* (***CAPN1***) gene encodes a cysteine protease, μ-calpain, that degrades myofibrillar proteins under postmortem conditions and appears to be the primary enzyme in the postmortem tenderization process (Koohmaraie, 1992; 1994; 1996). Regulation of μ-calpain activity has been correlated with variation in meat tenderness (Geesink and Koohmaraie, 1999). Bovine *CAPN1* has been mapped to the telomeric end of BTA29 (Smith et al., 2000a), and recently, a QTL for tenderness was found to be segregating in this region of BTA29 in two resource populations (Casas et al., 2000; Morris et al., 2001). The moderate effect (0.4 standard deviation) of this QTL on Warner-Bratzler shear force, a mechanical measurement of tenderness (AMSA, 1995) in the U.S. Meat Animal Research Center (**MARC**) population supported the evaluation of *CAPN1* as a candidate gene for tenderness within this population. Our objective was to determine if genetic variation within the candidate gene *CAPN1* could be associated with the meat tenderness values of two resource populations exhibiting meat tenderness QTL on BTA29.

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[2]Mention of trade names, proprietary products, or specific equipment does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that also may be suitable.

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Materials and Methods

Bacterial Artificial Chromosome Sequencing

A random shotgun library was prepared from a Bacterial Artificial Chromosome (**BAC**) clone (Smith et al., 2000a; Warren et al., 2000) containing the *CAPN1* gene by partial digestion of ~5 μg of BAC DNA, with the restriction enzyme *CviJ*I (0.3 units per μL) essentially as described (Gingrich et al., 1996). Briefly, the DNA was incubated with enzyme in a 50-μL volume for 20 min, the resulting DNA smear was separated on a 1% agarose gel, and fragments in the 1 to1.5 kb range were isolated using a commercial kit (Novagen, Madison, WI). The eluted fragments were ligated to a pBluescript vector (Stratagene, La Jolla, CA) that had been linearized with restriction enzyme EcoRV and treated with alkaline phosphatase. Ligated fragments were transformed into Top 10 competent cells (Invitrogen, Valencia, CA). Sequencing was performed on PCR-derived templates as described (Smith et al., 2000b), using T7 and T3 as universal sequencing primers on an ABI 3700 and ABI 377 sequencers.

The sequences from BAC subclones were analyzed with the sequence assembly algorithm Phrap (Ewing and Green 1998; Ewing et al., 1998) to detect overlaps and form contigs. The previously published (Smith et al., 2000a) partial sequence of the bovine *CAPN1* gene was included in the data set to seed formation of contigs surrounding the gene. Sequence comparisons of the contig sequences with human genomic sequence surrounding the human *CAPN1* gene (GenBank accession number NT_030106.2) and the *E. coli* genome were performed using BLASTN (Altshul et al., 1990;1997).

Animal Sequencing and Single Nucleotide Polymorphisms Identification

Primers (Table 1) for PCR amplification were designed from the *CAPN1* gene sequence (GenBank accession AF252504 and AF248054) to obtain overlapping DNA fragments of 1 to 2 kb in predicted length. Production of amplicons for direct sequencing was performed in a 20-μL volume with Amplitaq Gold or HotStar Taq polymerase enzyme following the protocols supplied by the manufacturers (Applied Biosystems, Inc.; Qiagen, Inc). Sequencing was performed using one-fourth reaction volumes with BigDye Terminator as recommended by the manufacturer (Applied Biosytems, Inc.) and analyzed on an ABI 3700 sequencer. Amplicons were sequenced with the amplification primers.

Sequence data were entered into the MARC database, base calls were made using Phred, and the sequences were aligned using Phrap (Ewing and Green, 1998; Ewing et al., 1998). Consed viewer was used to identify and tag single nucleotide polymorphisms (**SNP**) (Gordon et al., 1998) as described (Heaton et al., 2001). Genotyping was performed using a mass spectrometry-based MassArray system as suggested by the manufacturer (Sequenom, Inc., San Diego, CA), employing a PCR primer tailed with universal primer sequence in combination with a universal primer carrying a biotin tag as described (Stone et al., 2002). Polymerase chain reaction amplification for MassArray assays was carried out as outlined above except that reactions were carried out in 50-μL volumes.

Resource Populations

The MARC resource family used in this study has been previously described (Casas et al., 1998). Briefly, a half-sib family was developed using a Piedmontese × Angus sire. A total of 209 offspring were produced by matings primarily to MARC III dams (¼ Angus, ¼ Hereford, ¼ Pinzgauer, and ¼ Red Poll). Dams were artificially inseminated, and the offspring were born during the spring of 1995. Calves were weaned at an average of 200 d and raised from weaning to slaughter on a corn-corn silage diet. Steers were slaughtered at a commercial beef processing facility after 194 to 312 d on feed, and heifers were slaughtered after 207 to 287 d on feed. Meat tenderness was measured in all animals on the longissimus thoracis as Warner-Bratzler shear force (kg) at 3 and 14 d postmortem after belt cooking as described by Wheeler et al. (1998). This family was used to detect QTL based on differences between the two paternal alleles for growth, carcass composition, and meat quality traits (Casas et al., 2000; 2001).

The New Zealand AgResearch resource family used in this study was the progeny of a Limousin × Australian Jersey sire. The bull's semen was imported into New Zealand for insemination of New Zealand Limousin and New Zealand Jersey cows (mainly grade animals), thus providing backcross animals of two types for a joint New Zealand-Australian DNA marker study. Importation into New Zealand of semen from this and two other bulls from the Australian "Mapping Project" (Pitchford et al., 1998), provided common genetic links between New Zealand and Australian grandparents for the joint study. The cows in New Zealand were inseminated in 1995 and 1996, and calves were born in 1996 and 1997 in industry and research herds. Heifer and steer calves were collected after weaning and grown out on a research property (Tokanui Station) near AgResearch's headquarters. Further details of trial design and animal management (live calves and at slaughter) are given by Morris et al. (2001). Briefly, animals were preallocated to slaughter groups at 22 to 28 mo of age, over 18 kill days in 1998 and 10 kill days in 1999, with preallocation based on breed of calf, sire, and balanced as far as possible for live weight before the first slaughter day. The right striploin (longissimus lumbarum) was removed for tenderness measurement before stimulation of the remainder of the carcass. Each striploin was maintained at 15°C after removal. Five steak portions from the unstimulated striploin were cut for the cooking and shear force measurements with the first steak processed at rigor mortis and the remainder

Table 1. Primer pairs used for amplification of genomic ***CAPN1***

Forward primer	Reverse primer	Location in *CAPN1*[1]
CTGCTGCCCCTGGTTAAA	GGCCAAGCTGGGGGAACG	E1
GCCGAGGAGATACCGTGAA	CTGGCCCAGGTACTTGATGG	I1
GATCCTTCAACCCGTCTC	AGGTACTTGATGGCATTTTC	I1 partial, E2 partial
CTGATCCTTCAACCCGTCTC	GGTGCCCCTCAACTTACAGA	I1 partial, E2, I2, E3, I3 partial
GGTAGCATTGGGTGAGGGTA	AGTGCCATACTGGGGAGAAC	E2, I2, E3, I3 partial
GTTCTCAAACCCCCAGTTC	ATGTCTCTTTCCCAAAACGA	I3, E4, I4 partial
TGGCTACGCTGGCATCTTC	TTGCAAAGCCCTTGGACC	I4 partial
ATGGCTACGCTGGCATCTTC	GTGGGTCCCGTCAAGTGAA	I4 partial
ACTTGACGGGACCCACAGTG	GGAGGAGGGGCACAGTGAAT	I4 partial
CGCATTTTCATTCCACATCT	ACCGCTAGGAGACTCAGGTC	I4 partial, E5, I5, E6
GCTGTGGCAGTTTGGTGAGTG	CAGGGCCTTGAGGATGATGTT	E5 partial, I5 E6 partial
GCTGTGGCAGTTTGGTGAG	GAGAAGAGGGGTGAGCAGAG	E5 partial, I5, E6, I6 partial
TCTGAGGGCTTTGAGGACTT	ATCTGGCCCTGGTAGTTCAC	I6, E7, I7
CCCCGTGACCTTCAGCAGCAC	GTTGCGGAACCTCTGGCTCTTGAG	I7 partial, E8, I8, E9, I9
GGACTCTCGCCCAAAGAT	CCAGGTGCCCTCATACAG	I7 partial, E8, I8, E9, I9
GCTGTGCCATGTCTCTTGA	CTGGACAGCAAGGAAGTCTC	E10
CCCCTGGCTGCCTTAGTTCT	AGATCCCCTTTGCTCACTGG	E11
CCATAGGCTTCGCTGTCTAC	GGCCACGTGTGTTCAAG	I11, E12
TGGGCCCCTGTGTGG	ATGGGCAAACAATGGAAACA	E12, I12 partial
GCAGGCAGGGCTTTTACA	CTCTGAGAAGAAACGCAGCA	E13
GAGCCCAACAAGGAAGGT	AATACAGCCCAATGATGAGG	I13, E14, I14 partial
GGTCCGAGCAGTTCATCAAC	ACTTGCTGGAGAGGGAAGGT	I13, E14, I14 partial
GTCCAGGCCAATCTCCCC	AGCTCCTTGACGCTGATCTC	I14, E15, I16
TCATCATTGGGCTGTATTTCC	GCTGCGTCGGGTCTTG	I14 partial, E15, I15, E16, I16 partial
GACATGGAGATCAGCGTCAA	GCAGGGAAGGGGGTCAC	I16 partial
CCTCTTAAATTCCTCTTGCCAGAC	GAAAACTCCACAGCGTAAACCAG	I16 partial, E17, E17 partial
CCAGCACCGGTCCTTTTTAC	GAGGTAGGGGAGGGGAGAA	I16 partial, E17, E17 partial
TTCAGCCTGGAGTCCTGC	CATGCTGCCCGACTTGTC	I17, E18, I18
CTGGTTTACGCTGTGGAGTT	CCTGGCCTCATCCTGAA	E18, I18, E19, I19 partial
GCAGCATGAGTGCCTATGAA	CAGGCAGCACACGAAGTTG	I19
TCACCCTCACAGTCCCACTT	GGGCCACTCTTACCTCCTCT	I19 partial, E20, I20, E21
GACCTGGCCGTGGACTT	GCAGAGGAGCATAGCAAGG	I20, E21, I21
GGGCAGTGGGTTTTTCTCAC	GCCTCTGGTATCCCCGTAAC	I21 partial, E22 partial
CCTCTGCCCTCCTCGTC	CTGGTATCCCCGTAACTCAGT	E22 partial
TGACCATGTTTGCGTGAGAC	ATGTGGGACCTCAGCAGTGT	E22

[1]*CAPN1* = micromolar calcium-activated neutral protease, E = exon, I = intron.

at 1.3, 2.0, 2.3, and 4.0 d postmortem. A different technique for measuring shear force was used in the AgResearch population as shear force was measured by MIRINZ tenderometer (Fraserhurst and MacFarlane, 1983).

Haplotype Analysis

Haplotypes inherited from the sires were established based on a selection of 10 SNP representing genetic variation within the Piedmontese × Angus sire. Two SNP representing predicted amino acid changes, two SNP representing silent substitutions within the coding region, and six SNP representing intron variation were selected out of 38 total SNP heterozygous in the Piedmontese × Angus sire. Six of the SNP selected reside on the half of the gene 5′ to the approximately 100-kb intron 10, while the remaining four are located in the half 3′ to intron 10 (Figure 1). The haplotypes inherited by the sire from the Piedmontese grandsire and Angus grandam were identified by inferring the haplotypes based on offspring that were homozygous for all of the SNP tested and comparing these genotype patterns with markers used in the QTL analysis. All offspring from the MARC family were genotyped for the 10 SNP, offspring from the New Zealand family were genotyped for eight of these SNP informative in the Jersey × Limousin sire.

Dam Genotypes

Dam alleles were predicted based on the sire's haplotype and the SNP genotypes of the progeny. The effect of the allele inherited from the dam was estimated simultaneously with the effect of the paternally inherited haplotype in the MARC population using multiple regression of SAS (SAS Inst. Inc., Cary, NC). Variation associated with the maternally inherited alleles was partitioned separately from that associated with paternal alleles in order to isolate novel variation from previously reported differences associated with paternal alleles (Casas et al., 2000). The two SNP predicting valine or isoleucine (V^{530}/I^{530}) and glycine or alanine (G^{316}/A^{316}) were evaluated. The analysis provided least squares means by using a model that included the effects of sex (steers or heifers) and days on feed as a covariate.

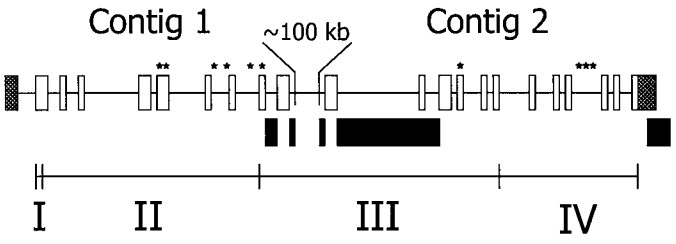

Figure 1. Schematic of the *calcium-activated neutral protease* gene. The boxes represent exon sequence and the connecting lines represent the intron sequence. The approximately 100-kb intron 10 is shown as a break in the sequence. The open boxes represent coding region and the hatched boxes represent untranslated sequence. The black boxes located below the schematic of the gene indicate sequence generated from this study. Contigs 1 and 2 from this study are indicated and contig 3 is located within the ~100-kb intron. The domains of the protein are marked by roman numerals I to IV and the set of 10 single-nucleotide polymorphisms (SNP) for haplotype construction are shown as stars. The first star on the left is SNP 1 followed by the rest of the SNP in numerical order. The first two SNP were included only in the U.S. Meat Animal Research Center quantitative trait locus population whereas 3 to 10 were included in both resource populations.

Results

Bacterial Artificial Chromosome Sequencing. The first step in the evaluation of *CAPN1* as a candidate was to obtain sufficient sequence to enable identification of SNP within the entire coding region of the bovine gene. Partial sequence (11,055 bp) of the bovine *CAPN1* gene (Smith et al., 2000a) was available; however, the reported *CAPN1* sequence excluded sequences between exon 9 and exon 11. To obtain this sequence, a subclone library was made from a BAC containing the *CAPN1* gene. Clones were sequenced from both ends to generate 5,947 sequence reads, a predicted 10-fold coverage of the BAC. Analysis of the sequences assembled 4,133 of the reads into 362 sequence contigs, with the remainder being low quality sequence reads or failing to show overlap with other sequences.

The three largest bovine contigs (containing 1,776 reads), were examined by BLAST analysis. The first contig contained exons 1 to 10 of the bovine *CAPN1* gene, the second contig contained exons 11 to 22 of the bovine *CAPN1* gene, and the third contig shared homology to the human *CAPN1* intron 10 (estimated to be approximately 100 kb based on the draft human sequence). The three contigs containing *CAPN1* genomic sequence represented a total length of 43,848 bp. No matches or overlaps were found from the remaining contigs that added sequence information.

The two contigs containing coding portions of the bovine *CAPN1* gene added significant new sequence, relative to the previous report by Smith et al. (2000a), within and around the exons as indicated by filled rectangles in Figure 1. Furthermore, two significant aspects of *CAPN1* gene architecture were revealed that had not been identified in the previous study. The additional sequence upstream of the 100-kb intron revealed a 130-bp intron in exon 9, that had not been detected by the exon primer-based PCR approach used previously (Smith et al., 2000a). Thus, the portion of the bovine *CAPN1* gene upstream from the 100-kb intron includes 10 exons, rather than the nine as previously reported. The second aspect involved the first intron downstream of the large intron which had been reported to be approximately 4 kb, but the new sequence data demonstrated that this intron is divided into a 2,791-bp intron, 12-bp exon, and an 891-bp intron (Figure 1). Therefore, the half of the gene downstream of the large intron includes 12 exons, rather than 10 as previously reported. In summary, the data indicate that the bovine *CAPN1* gene consists of 22 exons and 21 introns. This is the same arrangement observed for the human gene by comparison of the human cDNA (accession NM_005186) and the draft genome (Lander et al., 2001) sequences (data not shown). The updated sequence information was sufficient to support primer design to scan all exons of the gene. No effort was made to completely sequence the ~100-kb intron 10. In all, more than 28 kb of sequence containing the entire coding region of the bovine *CAPN1* gene was determined.

Single-Nucleotide Polymorphism Iidentification in MARC Quantitative Trait Locus Population. The hypothesis that *CAPN1* variation underlies the QTL effect observed in the MARC resource population predicts the presence of sequence variation between the two alleles of the Piedmontese × Angus sire of the population (**sire PA**). To identify this variation, a set of PCR primers was designed to amplify and sequence the coding region of the gene (Table 1). A total of 12.5 kb of the gene was encompassed by 35 amplicons. All 22 exons were completely sequenced to identify coding region variation, and all but the two largest introns were included to identify sufficient numbers of SNP to support construction of haplotypes. This procedure identified 38 SNP in sire PA, of which five were in exons. Two of the five exon SNP predicted amino acid variation in the μ-calpain protein, including a GCC alanine codon in exon 9 vs a GGC glycine codon (amino acid number 316), and a GTC valine codon in exon 14 vs an ATC isoleucine codon (amino acid number 530).

A set of microsatellite markers had been used previously to define the sire allele with effect on shear force measurement of meat tenderness. However, mapping the *CAPN1* gene to the QTL interval (Smith et al., 2000a) did not define which allele of the gene would associate with decreased meat tenderness (increased shear force), nor did the previous analysis establish haplotypes to permit this analysis. Therefore, the haplotypes of the sire were determined by genotyping of the progeny to identify homozygotes of each haplotype and permit unambiguous assignment of SNP alleles to

Table 2. Ten SNP used to generate the haplotypes for the two resource population sires, in the order of location starting from the 5′ end of the calcium-activated neutral protease gene[1]

SNP #	Sequence[2]	Location	MARC allele 1 (Piedmontese)	MARC allele 2 (Angus)	AgResearch allele 1 (Limousin)	AgResearch allele 2 (Jersey)
1	CAGCAC**R**TCTGAG	Exon 6	G	A	A	A
2	CACCGG**Y**GGAGTC	Exon 6	T	C	C	C
3	AGCTGC**Y**TCTCTC	Intron 7	T	C	T	C
4	GCTGGG**Y**TCTGTG	Intron 7	T	C	T	C
5	TGTGAC**Y**GGGTCT	Intron 8	C	T	C	T
6[3]	GGAACG**S**CGTGGA	Exon 9	G (Gly)	C (Ala)	G (Gly)	C (Ala)
7[4]	GACCAG**R**TCCAGG	Exon 13	A (Ile)	G (Val)	A (Ile)	G (Val)
8	GGTAAC**Y**GTTAGC	Intron 18	T	C	T	C
9	AGTACT**Y**TTGCCT	Intron 18	C	T	C	T
10	TTCCGA**R**CAGATG	Intron 18	G	A	G	A

[1]MARC = U.S. Meat Animal Research Center, SNP = single-nucleotide polymorphisms.
[2]R = A or G, Y = C or T, and S = C or G.
[3]Predicted glycine to alanine amino acid substitution.
[4]Predicted valine to isoleucine amino acid substitution.

each haplotype. This analysis demonstrated that one sire allele contained alanine at position 316 (allele A^{316}) and valine at position 530 (allele V^{530}), whereas the other sire allele contained glycine (allele G^{316}) and isoleucine (allele I^{530}) at those same two positions. A set of 10 SNP heterozygous in sire PA were chosen to genotype the entire resource population, to determine the haplotype associated with increased shear force. The SNP chosen and their alternative alleles in this bull are shown in Table 2, with the positions in the gene shown graphically in Figure 1. We arbitrarily define allele 1 of this sire as the GTTTCGATCG haplotype containing G^{316} and I^{530}, and allele 2 as the ACCCTCGCTA haplotype containing A^{316} and V^{530} with each base designation representing the allele of each SNP in the order shown in Table 2. Alleles 1 and 2 were inherited from the Piedmontese and Angus parent of the sire, respectively. Consistent with the original microsatellite-based QTL analysis, the haplotype analysis revealed that the Piedmontese allele had an effect on meat tenderness in this population (Table 3). This result suggests that the G^{316} I^{530} haplotype is a marker for a functional allele of *CAPN1* associated with increased shear force in this population.

An alternative hypothesis for the observed effect is that *CAPN1* markers are in linkage disequilibrium with a functional allele of another gene mapping to this part of BTA29. In this case, recombination will eventually break the association of the functional allele with *CAPN1* haplotypes. To test whether *CAPN1* alleles are the source of the meat tenderness variation, the contribution of alleles from the dams of the population were examined. If one or both of the predicted amino acid variations are markers for functional alleles with respect to shear force, then maternal inheritance should have a similar impact on phenotype. On the other hand, the association will be unlikely if the functional allele is in a different gene, since the dams are distantly related to the Piedmontese breed and an ample number of generations has occurred for recombination of the *CAPN1* alleles and putative functional alleles of other genes. The dam haplotypes were inferred by subtraction of the known sire haplotype from the genotypes of the calves, and analyzed simultaneously with the sire allele as a fixed effect. Table 3 summarizes the analyses of the sire alleles and the dam alleles with regard to shear force mean differences and their significance as measured by the *P* value. The magnitude of effect is measured by both shear force mean differences in the two alleles tested and the calculated effect in standard deviations. The dam alleles containing I^{530} or V^{530} were analyzed separately from the dam alleles containing G^{316} or A^{316} as numbers of dams containing inferred genotypes for both markers were insufficient for haplotype analysis. The G^{316} allele inherited from the dams was present at 86% frequency and did not show an effect on meat tenderness. The I^{530} allele was present in the dams at 18% frequency and showed an effect on meat tenderness (increase in shear force d 14, $P < 0.04$), providing strong support for the hypothesis that this SNP is in linkage disequilibrium with a functional allele.

Single Nucleotide Polymorphism Identification in AgResearch Quantitative Trait Locus Population. A second resource population at AgResearch in New Zealand had been developed that showed evidence of a QTL on BTA29 (C. Morris, unpublished data). A reciprocal backcross population using a Jersey × Limousin sire (**sire JL**) revealed a significant QTL approximately centered at the map position of *CAPN1,* affecting MIRINZ tenderometer shear force. This resource population had a different breed composition and was raised in a different environment (New Zealand vs Nebraska), by a different production protocol, suggesting it represented a rigorous test for the association of particular *CAPN1* haplotypes with meat tenderness across populations.

Table 3. Least squares mean, standard error, and probability values for meat tenderness measured as Warner-Bratzler shear force (kg) at 3 and 14 d postmortem

Alleles	Number of progeny[1]	Shear force d 3	Shear force d 14
Sire			
1 (Piedmontese)	83	4.73 ± 0.1 kg	3.52 ± 0.07 kg
2 (Angus)	94	4.38 ± 0.09 kg	3.26 ± 0.06 kg
Mean shear force difference		0.35 kg	0.26 kg
Effect		0.4 SD[2]	0.43 SD
P-value		0.0071	0.0046
Dam			
I^{530}	30	4.62 ± 0.16 kg	3.61 ± 0.1 kg
V^{530}	137	4.55 ± 0.08 kg	3.35 ± 0.05 kg
Mean shear force difference		0.08 kg	0.25 kg
Effect		0.09 SD	0.42 SD
P-value		0.68	0.034
Dam			
G^{316}	141	4.6 ± 0.08 kg	3.34 ± 0.05 kg
A^{316}	23	4.47 ± 0.2 kg	3.6 ± 0.13 kg
Mean shear force difference		0.13 kg	0.26 kg[3]
Effect		0.14 SD	0.42 SD
P-value		0.555	0.08

[1]Number of progeny inheriting indicated allele.
[2]SD = standard deviation.
[3]Note that the difference is in the opposite direction from all other shear force mean differences listed.

The *CAPN1* gene of the Jersey × Limousin sire was sequenced. No additional variation within the coding region was identified that would be predicted to alter the amino acid sequence of the protein. Genotypes from the 10 SNP used in the MARC population demonstrated that sire JL was heterozygous for eight of the 10, including the two predicting amino acid variation. Genotypes for the eight informative SNP were collected on the progeny of the resource population to define haplotypes of the bull and to determine which haplotype had the effect of increased shear force. The two haplotypes are shown in Table 2, with allele 1 arbitrarily defined as the allele containing G^{316} and I^{530}. Use of these SNP markers in a QTL analysis of the New Zealand population demonstrated that allele 1 of sire JL is associated with decreased meat tenderness (i.e., increased shear force, $P < 0.0001$) in this Jersey × Limousin cross (Table 4). Allele 1 was derived from the Limousin grandsire and is highly similar to allele 1 of the MARC sire, providing support for the hypothesis that these SNP represent markers of functional alleles of *CAPN1*.

The dam contributions for the two amino acid variants were inferred using the known sire contributions to the progeny. The shear force values as measured by the MIRINZ tenderometer test and results of the association analyses for both the dam and sire contributions to the progeny are summarized in Table 4. The analysis of the dam contributions revealed an effect for the G^{316} allele similar to that observed in the sire alleles of both resource populations. No effect was observed for the I^{530} allele when the dam contributions were analyzed as independent variables.

The identification of two μ-calpain variants that show effects on meat tenderness in unrelated QTL populations led to examination of the gene in a wider source of germplasm to ascertain the depth of *CAPN1* diversity in cattle. The same primers previously used to amplify overlapping portions of the gene were applied to sequence a sampling of 16 beef breeds and the Holstein dairy breed represented in the MARC bovine diversity panel (described in Heaton et al., 2001). An additional 134 SNP were detected in this panel, but none of the eleven present in exons were predicted to result in amino acid variation in the protein, nor were there any other obviously significant changes to the gene. The I^{530} allele was observed in all of the breeds in the diversity panel except Brangus and was present at an overall frequency of 30%. The G^{316} allele was observed in all of the breeds represented by the diversity panel and was present at an overall frequency of 86%. The A^{316} allele was not observed in Brahman, Simmental, Gelbvieh, Salers, Maine-Anjou, or Chianina breeds.

Discussion

We have characterized variation in the bovine *CAPN1* gene to identify markers for alleles affecting meat tenderness. In the Piedmontese × Angus sire of the MARC resource population, a haplotype was defined by 10 SNP that were representative of the variation observed by virtue of exonic and intronic SNP lying in both halves of the gene separated by an intron of over 100 kb. This group of SNP successfully determined the sire contribution to progeny, even in animals heterozy-

Table 4. Least squares mean, standard error, and probability values for meat tenderness measured as MIRINZ Tenderometer shear force (kg) at 1.3, 2.0, and 2.3 d postmortem

Alleles	Number of progeny[1]	Shear force (kg) at days postslaughter[2]		
		d 1.3	d 2.0	d 2.3
Sire				
1 (Limousin)	31	12.01 ± 0.36 kg	8.55 ± 0.24 kg	7.68 ± 0.19 kg
2 (Jersey)	50	9.75 ± 0.27 kg	6.86 ± 0.18 kg	6.33 ± 0.15 kg
Diff		2.26 ± 0.45 kg	1.69 ± 0.30 kg	1.35 ± 0.24 kg
Effect		1.13 SD	1.3 SD	1.27 SD
P value		3×10^{-6}	2×10^{-7}	3×10^{-7}
Dam				
I^{530}	16	11.36 ± 0.53 kg	8.08 ± 0.35 kg	7.20 ± 0.29 kg
V^{530}	58	10.77 ± 0.29 kg	7.72 ± 0.19 kg	6.99 ± 0.16 kg
Diff		0.58 ± 0.65 kg	0.37 ± 0.43 kg	0.20 ± 0.35 kg
Effect		0.27 SD	0.26 SD	0.17 SD
P-value		0.37	0.40	0.57
Dam				
G^{316}	39	11.51 ± 0.30 kg	8.02 ± 0.20 kg	7.37 ± 0.16 kg
A^{316}	34	10.12 ± 0.35 kg	7.29 ± 0.23 kg	6.58 ± 0.19 kg
Diff		1.38 ± 0.48 kg	0.73 ± 0.32 kg	0.79 ± 0.26 kg
Effect		0.70 SD	0.56 SD	0.75 SD
P-value		0.0049	0.026	0.003

[1]Number of progeny inheriting indicated allele.
[2]SD = standard deviation.

gous at multiple positions and in the absence of dam genotypes. The initial study indicated that the paternally inherited haplotype containing G^{316} and I^{530} was associated with increased shear force in the MARC resource population. This conclusion is limited, since these were the same animals as the original quantitative study and, therefore, the two sire alleles of any gene in the QTL interval would be expected to show contrast in the population. However, the dam alleles of the resource population represented an ideal resource to address the possibility that the observed *CAPN1* sequence variation represents useful markers for functional alleles affecting meat tenderness. The dams were from a different genetic background than the sire (Piedmontese × Angus sire vs Hereford, Angus, Pinzgauer, and Red Poll composite dams), making it unlikely that an association of phenotype with *CAPN1* markers in the dam alleles would occur as a result of population stratification or breed effect. Subtraction of the sire haplotype from the progeny genotypes was an efficient means to determine the dam allele contribution, even in the absence of DNA samples from the dams.

No association with decreased meat tenderness was detected for the G^{316} allele contributed from the dams in the MARC resource population, in contrast to the effect observed in the sire alleles. The low frequency of A^{316} in the dams resulted in many of the progeny inheriting a V^{530} and G^{316} allele from the dam which is out of phase with the inheritance pattern from the sire. The inheritance of these two opposing alleles from the dam limits the conclusion that can be made when an effect is not observed. The I^{530} allele, however, showed a similar effect on shear force when the dam alleles were analyzed as had been observed in the sire alleles, providing strong support for the utility of this marker. Further strong support is provided by the discovery that the I^{530} allele also is correlated with increased shear force in the AgResearch population, which is comprised of different breeds, raised on a separate continent, and with different management variables. The association of I^{530} with decreased tenderness in three different genetic backgrounds as demonstrated by the MARC sire (Piedmontese × Angus), the MARC dams (Red Poll, Pinzgauer, Hereford, Angus) and the AgResearch sire (Jersey × Limousin) provides strong evidence for I^{530} as a functional marker. The association of the G^{316} allele with increased shear force in the AgResearch population was demonstrated when inherited from either the sire or the dams providing support for the utility of this marker in addition to the I^{530} marker. The association of G^{316} with increased shear force is demonstrated in three different genetic backgrounds as demonstrated by the MARC sire (Piedmontese × Angus), the AgResearch sire (Jersey × Limousin) from Australia, and the AgResearch dams (Jersey and Limousin) from New Zealand.

Although differences were not detected in the AgResearch dams between the I^{530} and V^{530} alleles, the low frequency of dams contributing I^{530} in the AgResearch population may have limited the ability to detect a difference in shear force values. In addition, tenderness measurements were taken only up to d 4 postmortem in this population. It is possible that differences are only detectable at d 14 postmortem as demonstrated by the MARC resource population. Similarly the low frequency of A^{316} in the MARC resource population may

have hindered the detection of a difference between A^{316} and G^{316} alleles in the MARC dams.

The overall results provide strong evidence that I^{530} and G^{316} are informative markers for meat tenderness variation. Both I^{530} and V^{530} alleles were detected in all breeds present in the diversity panel except for Brangus (note the panel has only four Brangus bull samples, and the Brahman and Angus bull samples had both alleles), with no breed appearing fixed for the I^{530} allele. The observed frequency of I^{530} across the entire panel was 30%, sufficiently high to suggest that selection could impact variation in meat tenderness. The observed frequency of G^{316} across the entire panel was 86% with Brahman, Simmental, Gelbvieh, Salers, Maine-Anjou, and Chianina appearing fixed for the G^{316} allele. However, the low number of samples from each breed does not support the precise calculation of frequency within breeds.

In addition to utility as markers, it is possible that one or both of the amino acid variations reflect a mutation that causes a functional change in the μ-calpain protease. The μ-calpain isoform containing I^{530}, G^{316}, or both may be a functionally different protein that led to variation in myofibrillar proteolysis and resulted in a difference in shear force in the two QTL populations. The I^{530}/V^{530} variation represents a relatively conservative substitution of nonpolar amino acids in Domain III of the protein, which currently has no known function in terms of the activity of the enzyme. However, it could potentially alter protein folding or stability, impacting autocatalysis or proteolytic activity. Moreover, a major change in the protein is unlikely to be necessary to effect the relatively small change in postmortem proteolysis underlying the variation in shear force measurement. A peptide search using NCBI BLAST of bovine Domain III revealed that human, mice, rat, rabbit, chicken, and monkey versions of μ-calpain all have isoleucine at position 530. In contrast, sequences for swine, sheep, zebrafish, and frog all have valine at this position, which is the most common allele in the beef diversity panel. The region of comparison was well conserved among species (> 90%) except for chicken (66%) and zebrafish (56%). More sequencing of these other species is necessary before any conclusions could be made regarding these potential differences. Similar to the I^{530} allele, the variation represented by the G^{316} and A^{316} alleles represent a relatively conservative substitution of non-polar amino acids. However, this variation is located in Domain II of the protein, which has been identified as the proteolysis domain. An alteration in the proteolysis domain could alter the activity of the protein, and thus effect meat tenderization. A peptide search using the region around this variation revealed that monkey, human, mice, rat, chicken, and frog versions of μ-calpain do not contain glycine or alanine. The region in general is less conserved (< 90%) for the species mentioned, and the presence of glycine, alanine, serine, asparagine, lysine, and glutamic acid demonstrate that this position is not highly conserved among species.

Our results demonstrate the association of the I^{530} and G^{316} alleles with meat tenderness in two separate populations as well as in the dam alleles of the MARC population and the AgResearch population, and are consistent with the hypothesis that variation in *CAPN1* is responsible for the detection of the BTA29 QTL in the two resource populations.

Implications

This report presents evidence that single nucleotide polymorphisms determining amino acid variation of valine or isoleucine at position 530 and glycine or alanine at position 316 in the *micromolar calcium-activated neutral protease* gene, are markers for meat tenderness variation. If the alternative genotypes can successfully predict meat tenderness phenotypes in larger, unrelated populations, it would represent the first example of a deoxyribonucleic acid test for genetic merit directly related to this trait and would represent a valuable tool for cattle breeders to improve tenderness in cattle.

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