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SHORT COMMUNICATION

Molecular cloning and mapping of the bovine and ovine skeletal muscle-specific calpains[1]

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Summary

The coding regions of the bovine and sheep skeletal muscle-specific calpains (*CANP3* or *p94*) were cloned and sequenced by RT-PCR. Direct sequencing confirmed open reading frames of 2466 bp for both species, and bovine and sheep CANP3 shared 98·5% identity in their amino acid code. These sequences were greater than 88% identical to human, pig, rat and mouse *CANP3* nucleotide sequences, and greater than 93% identical for the amino acid code. Single nucleotide polymorphisms were used to map the bovine and sheep *CANP3* genes in two steps. The genes were placed into linkage groups based on two-point LOD scores (≥ 3·0) and the best order was determined with multipoint linkage analysis (CRI-MAP vs. 2·4). Bovine *CANP3* mapped to bovine chromosome 10, relative position 33·9 cM with linkage to nine markers; LOD scores ranged from 4·89 to 8·61 (order, *BMS2349-BL1035-RME25-CANP3-BM6305-BMS861-ILSTS053-BMS2742-CA090-BMS529*). Ovine *CANP3* mapped to chromosome 7, relative position 58 cM, with linkage to only one marker, *BMS861* (a bovine microsatellite that has been used in sheep), with no recombination and a LOD score of 5·72. The observed heterozygosity was 50% for both *CANP3* markers in bovine and sheep pedigrees.

Keywords: bovine, CANP3, chromosomal location, coding region, ovine, p94, skeletal muscle-specific calpain

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[1]Mention of a trade name, proprietary product or specific equipment is necessary to report factually on the available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Calpains (calcium-activated neutral proteases; EC 3·4.22·17) are cytosolic cysteine endopeptidases that require calcium for catalytic activity. The ubiquitous calpains m-and μ-calpain (indicating their molar calcium requirements for proteolytic activity) consist of distinct large subunits (80K) and a common regulatory smaller subunit (28K), which are encoded by different genes on different chromosomes (Ohno *et al*. 1989). A number of physiological functions for calpains have been suggested (Molinari & Carafoli 1997; Saido *et al*. 1994) although their regulation *in vivo* is poorly understood. Tissue-specific calpains include skeletal muscle-specific calpain (Sorimachi *et al*. 1989) and stomach-specific calpain (Sorimachi *et al*. 1993a). Recently, subfamilies of calpains have been discovered with different tissue specificities, and are so far only classified by their sequence homology and tissue distribution (Dear *et al*. 1997).

The skeletal muscle-specific calpain (CANP3; calcium activated neutral protease, or p94, based upon its predicted molecular size) is expressed in skeletal muscle at appreciable levels, but the protein itself is nearly undetectable, presumably because of rapid autolysis after translation (Sorimachi *et al*. 1993b) making it difficult to study *in vitro*. In humans, mutations in the *CANP3* gene cause a form of limb-girdle muscular dystrophy (Richard *et al*. 1995). Antisense treatment of CANP3 in rat myoblasts leads to myofibrillar disorganization (Poussard *et al*. 1996). These findings suggest that CANP3 is important in muscle development and growth. The coding sequences for *CANP3* have been reported for human, mouse, rat and pig (Sorimachi *et al*. 1989; Richard *et al*. 1995; Briley *et al*. 1996; Richard & Beckman 1996). In this study, we report the *CANP3* coding sequences and chromosomal locations for bovine and sheep.

Total RNA was isolated from bovine and ovine skeletal muscle (Chomczynski & Sacchi 1987; Puissant & Houdebine 1990), selected for poly(A)$^+$ mRNA, and reverse transcribed (Clonetech, RT Advantage). An oligo (dT_{18}), or poly-T–anchored oligo (primer TRT designed for 3′ RACE) was used to prime the reverse transcrip-

tion. Primers designed from human, rat, mouse and pig sequences upstream of the initiation codon (SkmF2) and in the 3′ coding region (SkmR2 and SkmR3) were used to amplify the full-length 2·5 kb coding sequences of bovine and ovine *CANP3* (Table 1). Nucleotide sequences were extended to the poly A tail of the sheep *CANP3* transcript, and into over 400 bp of the bovine 3′ UTR by 3′ RACE. Amplicons were sequenced in both directions using internal primers (Table 1) to determine the entire coding sequence according to Geesink *et al*. (1998). These sequences have greater than 88% identity to other species (human, rat, mouse and pig) at the nucleotide level and greater than 93% homologous for the amino acid sequence (GenBank nos. AF087569 and AF087570). The bovine and sheep amino acid sequences are 98·5% identical. Nucleotide identity of the 3′-untranslated region of bovine and sheep was 96% over a 423 base pair overlap. Direct sequencing confirmed 2466 bp of open reading frame for both species, with a predicted molecular weight of 94·6 kDa for the coded protein. The sequences surrounding the initiator methionine were conserved with other species, and closely followed the Kozak consensus sequence (gccATGc; Kozak 1996).

Specific residues of the active site of calpains were conserved in the bovine and ovine CANP3 sequences; the cysteine at position 129, the histidine at position 335, and the asparagine at 359 (Sorimachi *et al*. 1989). Amino acid conservation in the NS, IS1 and IS2 regions of bovine and ovine CANP3 was greater than 97%, 97% and 92% homologous to each other, respectively. Also present in the bovine and sheep sequences is the nuclear localization signal found in IS2 (Roberts 1989).

Single nucleotide polymorphisms (SNPs) were used to map the bovine and sheep *CANP3* genes in two steps. The genes were placed into linkage groups based on two-point LOD scores ($\geq 3{\cdot}0$) and the best order was determined with multipoint linkage analysis (CRI-MAP vs. 2·4, Green *et al*. 1990). Bovine *CANP3* was mapped using a diallelic marker in intron 2 (GenBank accession no. AF087571) amplified from genomic DNA (obtained from MARC, USDA, Reference Population, bovine 4-way cross pedigree; Bishop *et al*. 1994). A PCR/RFLP assay was devised to amplify a 207-bp fragment in intron 2 to distinguish a *C*-allele or deletion allele (128 bp from exon 3) using forward primer SkmF18 and reverse primer SkmR12. The reverse mismatch primer created an *Xcm*I restriction site near the polymorphism that would distinguish between the two alleles, where the *C*-allele was digested, and the deletion allele destroyed the restriction site. Digests of this amplicon yielded 207 bp (deletion) or 186 and 20 bp (*C*-allele) fragments. Bovine *CANP3* mapped to bovine chromosome 10, relative position 33·9 cM with linkage to nine markers; LOD scores ranged from 4·89 to 8·61 (order, *BMS2349-BL1035-RME25-CANP3-BM6305-BMS861-ILSTS053-BMS2742-CA090-BMS529*). The observed heterozygosity was 50% in this pedigree. Linkage and marker information can be obtained from the web site http://sol.marc.usda.gov/marc/html/gene1.html.

Ovine *CANP3* was mapped using a diallelic marker located in exon 1 of the *CANP3* gene. PCR products were amplified from genomic

Table 1. Primer sequences used to amplify and sequence bovine and ovine *CANP3*

Primer	Nucleotide sequence (5′ > 3′)	Direction	Position
SkmF2	gct tcc ttt cct tga agg tag ctg	forward	− 69– − 44
SkmF7	act aca gag gcc atg gag gac ttc ac	forward	671–695
SkmF8	gga cga gaa ggc ccg ttt gca gca cc	forward	1146–1170
SkmF10	cag ttt cat ctg ctg ctt tgt cag gc	forward	2343–2368
SkmF11	caa tgc agt caa aga tgc agg ctt cc	forward	2246–2275
SkmF13	aca atc tct gtg gat cgg cca gtg aa	forward	1762–1787
SkmF18	ggc tga cat gtc cgt aag gtc aga tc	forward	intron 2
SkmR1	tag gtt ggc agg cag tca tc	reverse	554–535
SkmR2	cat ctg gtt cag gca tac atg gta ag	reverse	2474–2452
SkmR3	ctg gtt cag gca tac atg gta agc tg	reverse	2471–2449
SkmR6	aac tcc ccc att ttc agc cca gaa gg	reverse	857–832
SkmR8	ctg aaa ata ttc cgg aat tgc cgc tg	reverse	1979–1954
SkmR11	ctg ctc gaa tgt ctt ctc ttt cac cc	reverse	233–258
SkmR12	gac gct tgt tca agg tca ggc aag cg	reverse	intron 2
TRT	atg cag aag tac atg ccg ta $t_{(17)}$	reverse	*
TR1	atg cag aag tac atg ccg ta	reverse	*

*Positions of these primers are undetermined. Positions of primers are numbered according to bovine GenBank sequence AF087569.

DNA (obtained from MARC, USDA, Reference Population, sheep pedigree; de Gortari *et al.* 1998) using a forward primer from the 5′-untranslated region (primer SKMF2) and a reverse primer (primer SKMR11) in exon 1 (Table 1). A polymorphic *T* or *G* allele in codon 15 (Ser or Ala) produced a *Tth111*I restriction site in this 258 bp amplicon with the *T*-allele. Restriction digest with *Tth111*I yielded 149 bp and 109 bp fragments. Ovine *CANP3* mapped to chromosome 7, relative position 58 cM, with linkage to one marker, *BMS861*, with no recombination and a LOD score of 5·72. Observed heterozygosity was 50% for this marker in the MARC pedigree.

The addition of the cDNA sequences of the bovine and ovine *CANP3*, and the chromosomal locations of this anchored type I marker will assist physiological and genetic studies on the role of CANP3 in muscle development in these species.

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