

Cloning and Characterization of Bovine Titin-cap (TCAP) Gene

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ABSTRACT : Titin-cap (TCAP), one of the abundant transcripts in skeletal muscles, was investigated in this study in cattle because of its role in regulating the proliferation and differentiation of myoblasts by interacting with the myostatin gene. From the 5' and 3' RACE experiments, full-length TCAP coding sequence was identified, comprising 166 amino acids. The amino acid comparison showed high sequence similarities with previously identified human (95.8%) and mouse (95.2%) TCAP genes. The TCAP expression, addressed by northern blot, is limited in muscle tissues as indicated by Valle et al. (1997). The radiation hybrid analysis localized the gene on BTA19, where the comparative human and porcine counterparts are on HSA17 and SSC12. A few muscle-related genetic disorders were mapped on HSA17 and some growth-related QTLs were identified on SSC12. The bovine TCAP gene found in this study opens up new possibilities for the investigation of muscle-related genetic diseases as well as meat yield traits in cattle. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 10 : 1344-1349)

Key Words : RACE, Northern Analysis, Radiation Hybrid Mapping, Cattle, TCAP

INTRODUCTION

Titin-cap (TCAP), also called telethonin (TELE), is a 19 kDa sarcomeric protein that was initially identified from human expressed sequences tags (ESTs). The TCAP transcript was shown to be among the 12 most abundant in skeletal muscles (Valle et al., 1997). It was believed that the TCAP gene was involved in the reorganization of the cytoskeleton during myofibrillogenesis and myofibril turnover (Mayans et al., 1998). Also, TCAP has been shown to be necessary for the structural integrity of sarcomeres (Gregorio et al., 1998) and a model has been proposed whereby TCAP acts to link the myofibrillar apparatus to the sarcolemma within the Z-line region (Furukawa et al., 2001).

Recently, Nicholas et al. (2002) identified the specific interaction between myostatin and TCAP proteins using yeast two-hybrid system. The myostatin gene belongs to the transforming growth factor β (TGF- β) superfamily of multifunctional polypeptide growth factors that are involved in the regulation of myoblast growth and differentiation. Evidence of myostatin as a key negative regulator of skeletal muscle growth has been well established. Inactivating mutations in myostatin gene result in an increase in muscle mass in double muscled cattle breeds such as Belgian Blue and Piedmontese (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997).

Therefore, the TCAP gene can regulate the proliferation and differentiation of myoblasts by controlling the amount of circulating active mature myostatin (Nicholas et al., 2002).

In this paper, we present a study of full-length TCAP gene sequence in cattle, its predicted amino acid sequence compared with other species and mapped the gene with 5,000 rad bovine radiation hybrid (RH) panel. Since the TCAP is highly correlated with myostatin gene in cattle, this gene can be used as a very valuable marker for muscle growth and yield.

MATERIALS AND METHODS

Genomic DNA preparations

Blood and skeletal muscle samples were collected from the Korean cattle bred in National Livestock Research Institute (NLRI) in Korea. Fresh bovine blood was collected in tubes containing heparin anticoagulant and suspended in a RBC (red blood cell) lysis buffer. White cells were collected by centrifugation and genomic DNA was extracted using a Mag Extraction Kit according to the manufacturer's instructions (Toyobo, Japan).

Total RNA preparations

Skeletal muscle tissues, heart, spleen, liver and kidney were sampled within 20 min of dissection. The sampled tissues were cut into small pieces and immediately emerged into liquid nitrogen and then stored in a -70°C fridge until use. One hundred mg of each sample tissue were homogenized in Tri Reagent (Molecular Research Center, INC. USA) using a high-speed mechanical homogenizer. Total RNA integrity was monitored by denaturing 1% agarose gel electrophoresis. RNA concentrations and purities were measured by spectrophotometer.

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Table 1. Nucleotide sequences and their positions of oligonucleotide primers used in this study

Identification	Sequences	Nucleotide sequence position*
BTEL-F	5'-GTGGAAGGATCTGACGCTGT-3'	102-121
BTEL-R	5'-AACTCAGGAGATCCAGGCAT-3'	903-922
BTEL-1F	5'-AGCGGCATGAGACCTATCACCGGCA-3'	395-419
BTEL-2F	5'-GGCACCAAGGAGGAGCGCGAGGAGA-3'	556-580
BTEL-3R	5'-AGACATGGAGCGAGACAGGGAGCGA-3'	714-738
BTEL-4R	5'-GAGTCACGGTCCTCTCAGCCTCTCT-3'	748-774

* The primer positions are the nucleotide sequence positions in Figure 2 (B).

cDNA synthesis

cDNA was synthesized using SMARTTM RACE cDNA Amplification Kit (BD Clontech Biosciences, Palo Alto, CA, USA). PowerScriptTM Reverse Transcriptase was used and primed with 5'-CDS (5'-RACE codon), 3'-CDS (3'-RACE codon) and SMART II oligonucleotide primers (BD Clontech Biosciences, Palo Alto, CA, USA) to generate a complete cDNA copy of the 5' and 3' ends of the TCAP mRNA. The reaction consisted of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 6 mM MgCl₂, 2 mM DTT, 1 mM each of dATP, dCTP, dGTP and dTTP, 1 μM primer with 1 μg of total RNA from skeletal muscle. cDNA synthesis reaction was incubated at 42°C for 1 h 30 min.

PCR, rapid amplification cDNA ends (RACE) and cloning

The first strand cDNA products were diluted 10 times using Tricin-EDTA buffer [10 mM Tricine-KOH (pH 8.5) and 1.0 mM EDTA]. For both 5' and 3' RACE reactions, we carried out nested PCR reactions. The BTEL-3R and BTEL-4R primers were used as 5'-RACE reactions and the BTEL-1F and BTEL-2F primers were used as 3'-RACE reactions (Table 1). The final composition of the reaction consisted of 40 mM Tricine-KOH (pH 8.7), 15 mM KOAc, 3.5 mM Mg (OAc)₂, 3.75 μg/ml BSA, 0.005% Tween 20, 0.005% Nonidet-P40, 200 mM each of dATP, dCTP, dGTP and dTTP, 10 μM primers, *Taq* DNA polymerase (BD Clontech Biosciences, Palo Alto, CA, USA) with cDNA template. The thermal profile included 30 cycles of denaturation at 94°C for 30 sec, annealing at 68°C for 30 sec and extension at 72°C for 3 min. The obtained PCR products were analyzed on 1% agarose gels stained with ethidium bromide. PCR products were purified using NucleoTrap[®] gel extraction kit (BD Clontech Biosciences, Palo Alto, CA, USA), cloned into pGEM-T easy vector (Promega, USA) and transformed into *Escherichia coli* DH5α high efficiency competent cells to maintain the recombinant DNA. DNA sequences of clone inserts were determined using Applied Biosystems dye terminator kit on an ABI377 DNA sequencer (PE Applied Biosystems, USA).

Radiation hybrid analysis

A 380 bp product containing a part of exon 2 of the bovine TCAP gene was yielded from the PCR using BTEL-

1F and BTEL-4R primers. The PCR product was also sequenced to confirm whether the correct target PCR product was amplified. The chromosomal localization of the bovine TCAP gene was detected by PCR analysis of a bovine whole genome radiation hybrid panel (Womack et al., 1997).

PCR results were analyzed using the RHMAP (Ver 3.0, Boehnke, 1992) and RHMAPPY program (Ver 1.22, Slonin et al., 1997), which is located at the interpreting web pages at Texas A & M University (<http://bovid.cvm.tamu.edu/cgi-bin/rhmapper.cgi>). Using the two-point analysis in RHMAPPY program, the TCAP gene was assigned in a cattle chromosome in comparison with previously assigned anchor markers. Then, multipoint linkage analysis was performed using the RHMAP program. The map position of the TCAP gene was given in relation to markers in the first generation whole genome comparative map of cattle and humans (Band et al., 2000).

The 380 bp PCR product was amplified in a reaction containing 25 ng of template DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 mM MgCl₂, 0.2 μM of each primer, 100 μM of each dNTP and one unit of *Taq* polymerase (Promega, USA). The thermal profiles included an initiation denaturation at 94°C for 5 min, following 35 cycles of denaturation at 94°C for 30 sec, annealing at 68°C for 30 sec and extension at 72°C for 40 sec and then a final extension step at 72°C for 10 min using a PTC-200 Programmable Thermal Controller (MJ Research, Inc., USA).

Determination of gene organization

Based on comparison with the human TCAP sequences, TCAP gene was expected to have only one intron between exons 1 and 2. To determine the intron size of TCAP gene, PCR was performed with the primers, BTEL-F and BTEL-R, which are located on exons 1 and 2, for flanking the intron. The resulting PCR product was cloned and sequenced with the same procedures explained previously.

Northern blot

RNA samples were separated by electrophoresis in a 1% denaturing agarose gel and transferred to positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). A TCAP PCR product of 700 bp was random-

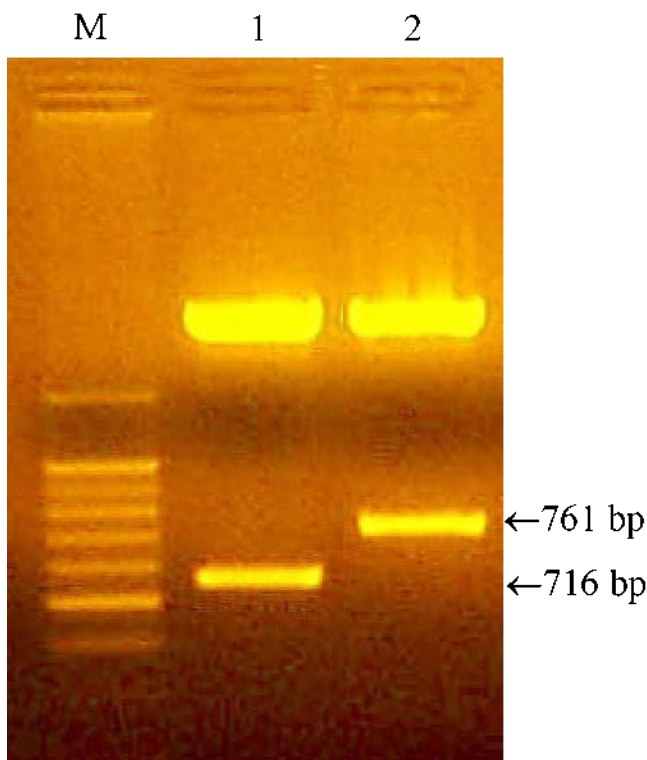


Figure 1. A 1.5% agarose gel showing the cloned TCAP RACE products digested with *EcoRI* restriction enzyme. Lane M is a 100 bp molecular marker (Promega, USA), lane 1: a 716 bp of 5' RACE fragments, lane 2: a 761 bp of 3' RACE fragments.

primed labeled with ³²P (Amersham Pharmacia Biotech, Buckinghamshire, England) as a probe for hybridization. After prehybridization at 42°C for 3 h, the labeled probe was denatured and transferred on to the membrane. Hybridization was carried out overnight at 42°C. Following hybridization, the membrane was washed and exposed to X-ray film.

RESULTS AND DISCUSSION

Cloning and sequencing of the TCAP gene

The Korean cattle TCAP ESTs (GenBank accession numbers: BM929201 and BM967971) were initially used as a template for the sequence searches against GenBank to extract all the lodged TCAP sequences. These TCAP ESTs were also subjected to aligned EST sequence search against TIGR (The Institute of Genome Research, <http://www.tigr.org>) database. As a result, an 898 bp of the putative TCAP aligned EST sequence was identified (TC204761). This sequence was used for designing primers for the RACE experiments (Table 1).

After verification by *EcoRI* restriction digest, a 716 bp fragment was amplified by 5' RACE using a RNA sample isolated from the Korean cattle skeletal muscle (Figure 1, lane 1). Sequence analysis revealed that this fragment

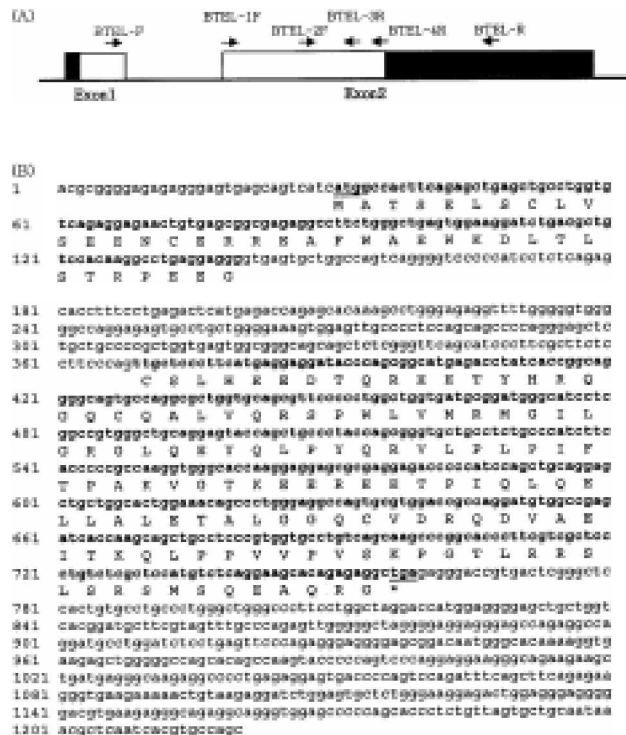


Figure 2. Gene structure and coding regions of bovine TCAP gene. Panel A: Schematic representation of bovine TCAP gene organization. The primer locations used in this study are indicated and 5' and 3' untranslated regions (UTRs) are shaded. Panel B: Nucleotide sequences and deduced amino acid sequences of the bovine TCAP gene. Translated exons are bolded, the atg start and tga stop codons are bold-underlined.

contained exon 1, part of exon 2 and a putative promoter region (Figure 2). For the 3' RACE, a complete 3' end sequence of 761 bp product was obtained including part of exon 2 and the polyadenylation signal (Figure 1, lane 2 and Figure 2). Thus, a complete mRNA transcript was identified consisting of 992 nucleotides with a 501 bp coding regions. Northern blot hybridization showed a bovine TCAP mRNA transcript of about 1 kb in size, in agreement with the results of the sequence analysis (Figure 4).

For amplifying unidentified gene sequences in one species, the orthologous gene information from other species is valuable. Previous comprehensive attempts to make cross-species primers have been reported by Venta et al. (1996) for Universal Mammalian Sequences-Tagged Sites (UM-STSS), Lyons et al. (1997) for Comparative Anchor Tagged Sequences (CATS), and Jiang et al. (1998) for Traced Orthologous Amplified Sequence Tags (TOASTs). However, the above attempts were limited by the design of their primers as they were based on a veiled knowledge of target sequences. To circumvent this limitation, the primer sequences were designed from aligned bovine EST sequences, which is a bovine-specific sequence resource. Successfully amplified partial TCAP

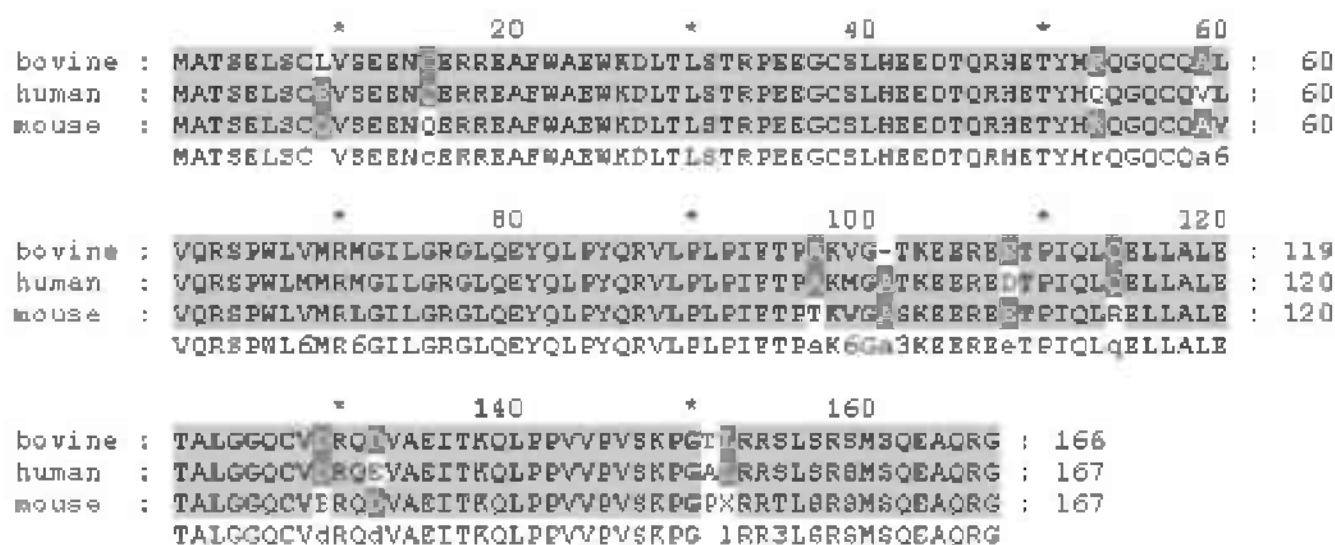


Figure 3. Comparisons of deduced TCAP amino acid sequences of bovine, human and mouse, showing similarities among species. The level of amino acid conservation among species are shaded at different degree.

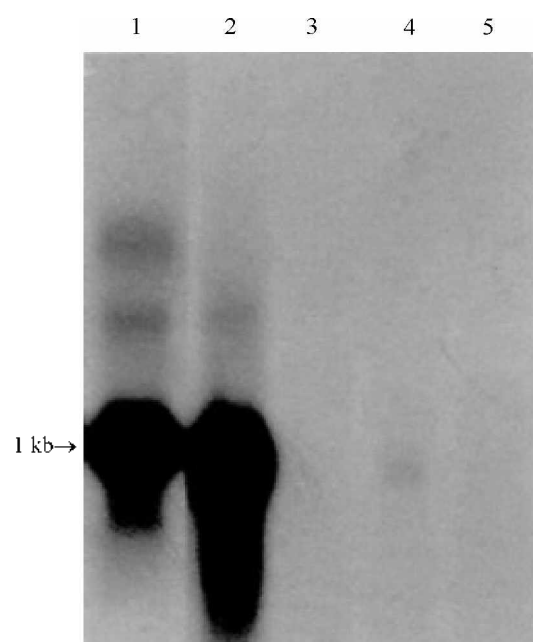


Figure 4. Northern blot analysis of TCAP expression in various bovine tissues. Lane 1: skeletal muscle, lane 2: heart, lane 3: liver, lane 4: spleen, lane 5: kidney.

gene in this study is a good example of the invaluable utility of ESTs, and enormous numbers of ESTs from various tissues have already been generated in livestock species (Smith et al., 2001; Takasuga et al., 2001; Band et al., 2002; Fahrenkrug et al., 2002; Pascual et al., 2002; Rink et al., 2002; Smith et al., 2002; Sonstegard et al., 2002).

Genomic structure of TCAP

A total of 1,220 bp nucleotides were obtained for the bovine TCAP gene and this sequence has been deposited at

GenBank (Accession number: AY428575). The deduced amino acid sequence of TCAP gene comprises 166 amino acids. The bovine TCAP gene was organized with one intron separating the two exons as in human and mouse (Figure 2). Splice donor and acceptor consensus sequences were identified at the exon/intron boundaries. A typical polyadenylation signal was located 25 bp upstream from the poly-A tail. Exon 1 consisted of 140 bp and encoded 37 amino acids and exon 2 consisted of 852 bp and encoded 129 amino acids, followed by a TGA stop codon. Between exon 1 and exon 2, a 228 bp of intron 1 is located (Figure 2). Comparison of bovine TCAP amino acids sequences with human and mouse showed 95.8 and 95.2% sequence similarities, respectively (Figure 3).

Radiation hybrid mapping

The analysis of 5,000 rad bovine radiation hybrid panel (Womack et al., 1997) showed the most significantly linked markers were U08018 (LOD score 17.7) and PPP1R1B (LOD score 16.2) on bovine chromosome 19 (BTA19) (Band et al., 2000). The assignment of bovine TCAP gene to BTA19 is consistent with the comparative chromosomal homology between human chromosome 17 (HSA17) and BTA19 (Band et al., 2000). Unfortunately, not many bovine QTL (Quantitative Trait Locus) results are available at present, especially for the meat yield traits; therefore, it is difficult to assume that the TCAP gene is a strong positional candidate gene. On the other hand, when we investigated the porcine comparative chromosomal location, pig chromosome 12 (SSC12) is the porcine counterpart of HSA17 (Lee et al., 2003). Compared with all identified porcine QTLs, SSC12 harbors QTLs for daily gain, birth weight and live weight (Bidanel and Rothschild, 2002).

Therefore, we can propose that the TCAP is a possible positional candidate gene for meat yield in cattle. In addition, there are several genetic disorders mapped onto HSA17, including some responsible for myopathies such as malignant hyperthermia susceptibility-2 and adhalinopathy (Levitt et al., 1992; McNally et al., 1994). The localization and characterization of the bovine TCAP gene in this study will give new information for investigation on the molecular characterization of these myopathic genetic diseases as well as the economically important meat yield traits.

Northern blot analysis

The levels of expression of the Korean cattle TCAP transcript seem to be restricted to skeletal and heart muscles under normal exposure conditions, as it cannot be detected in any other tissues including liver, kidney and spleen (Figure 4). The tissue specificity of TCAP transcript was also studied in several tissues by northern blot analysis and RT-PCR (Reverse Transcription-Polymerase Chain Reaction), as reported by Valle et al. (1997) and Mason et al. (1999). Further verification of the TCAP promoter regions will give useful clues for the muscle specificity in expression in mammals.

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