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ALTERNATIVELY POLYADENYLATED CALPASTATIN TRANSCRIPTS IN BOVINE MUSCLES

TRANSCRIPTOS ALTERNATIVAMENTE POLIADENILADOS DE CALPASTATINA EN MÚSCULOS DE BOVINO

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ABSTRACT

Calpastatin activity has a key role in the tenderization process that occurs during *post-mortem* storage of meat under refrigerated conditioning. The regulation of calpastatin (*CAST*) expression is highly complex, the gene has four putative promoters and at least three different polyadenylation sites, and it is also alternatively spliced. We investigated the presence of alternative polyadenylation (APA) isoforms of *CAST* transcripts in three muscles (*infraspinatus*, *triceps brachii* and *semitendinosus*) of two bovine breeds (Angus and Brahman). The 3´ RACE-PCR was used to specifically amplify the different APA sites. The amplified fragments were cloned and sequenced. Sequencing confirmed the existence of three expected polyadenylation sites corresponding to short, medium and long polyadenylated transcripts. Also, transcripts with a novel APA site were found in the three muscles of both breeds. Because the same APAs isoforms were found between muscles and breeds, we could hypothesize a possible contribution to the relative abundance of different isoforms, probably in coordination with promoter preference and alternative splicing. This knowledge would be useful in the design of future experiments to analyze differential expression of CAST isoforms and their contribution to the definition of beef tenderness.

Key words: Beef cattle, Alternative polyadenylation, 3´ RACE-PCR

RESUMEN

La actividad de la calpastatina tiene un rol clave en el proceso de tiernización *postmortem* de la carne durante su almacenamiento refrigerado. La regulación de la expresión de calpastatina (*CAST*) es altamente compleja; el gen tiene cuatro potenciales promotores, diferentes sitios de poliadenilación de transcriptos y también *splicing* alternativo. En este trabajo se investiga la presencia de isoformas de transcriptos de *CAST* alternativamente poliadenilados (APA) en tres músculos (*infraspinatus*, *triceps brachii* y *semitendinosus*) de dos razas bovinas (Angus y Brahman). Se utilizó la técnica de 3´ RACE-PCR para amplificar específicamente los diferentes sitios APA. Los fragmentos amplificados fueron clonados y secuenciados. La secuenciación confirmó la existencia de tres sitios de poliadenilación conocidos. Un nuevo sitio APA fue identificado en transcriptos de los tres músculos y en ambas razas. Dado que cualitativamente no hubo variación en la presencia de isoformas definidas por APA entre músculos y razas de terneza contrastante, podría hipotetizarse una posible contribución a la abundancia relativa de distintas isoformas, probablemente en forma coordinada con la elección de promotores y el *splicing* alternativo. Este nuevo conocimiento podría ser de utilidad para el diseño de experimentos de análisis de expresión diferencial de isoformas de calpastatina, para ponderar la contribución de las mismas a las variaciones en terneza de la carne.

Palabras clave: Bovinos para carne, Poliadenilación alternativa, 3´ RACE-PCR

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INTRODUCTION

The calpain/calpastatin system is an endogenous, calcium-dependent proteinase system. Calpain is involved in the breakdown of protein myofibrils; calpastatin inhibits calpain activity and, therefore, partially regulates *postmortem* proteolysis in muscle (Koohmaraie *et al.*, 1996). This enzyme complex affects some meat quality traits; particularly it plays an important role in meat tenderization (Shackelford *et al.*, 1995). *Bos indicus* breeds (*e.g.* Brahman) are well known for their higher calpastatin activity in muscle, which inhibits protein degradation and results in tougher beef (Whipple *et al.*, 1990; Pringle *et al.*, 1997).

The bovine calpastatin (*CAST*) gene consists of 35 exons spanning at least 130 kb on chromosome 7 (Bishop *et al.*, 1993; Raynaud *et al.*, 2005a). Four alternative promoters direct the expression of four different transcripts isolated from different tissues, named Type I, II, III, and IV, which differ in their 5´ ends (Raynaud *et al.*, 2005a). Moreover, differences in transcript length can also be originated by alternative polyadenylation sites and alternative exon splicing (Cong *et al.*, 1998; Raynaud *et al.*, 2005b; Nattrass *et al.*, 2014).

The polyadenylation (poly A) reaction of mammalian pre-mRNAs proceeds in two stages: first the cleavage of pre-mRNA and then the addition of poly(A) tail to the newly formed 3´ end. Polyadenylation is important for translation efficiency, stability, and cellular localization of mature mRNA (Elkon *et al.*, 2013). Many eukaryotic genes contain more than one polyA (pA) site, leading to the generation of distinct mRNA isoforms from the same gene through alternative polyadenylation (APA) (Tian *et al.*, 2017). The APA sites of *CAST* are located in the 3′ untranslated region (3′UTR), leading to alternative transcripts, all with the same coding frame but with variable 3′UTRs (called UTR-APA). It should be noted that although the UTR-APA isoforms do not affect the coding frame, they might lead to changes in mRNA half-life or translation efficiency, since longer 3′UTRs can have more microRNA binding sites, more RNA-binding protein recognition sites, or altered RNA secondary structure (Millevoi and Vagner, 2010; Mayr, 2016).

Three polyadenylated variants in the 3´ UTR have been described for bovine *CAST* transcripts, named: short, medium and long (Cong *et al.*, 1998; Raynaud *et al.*, 2005b).

The medium form is 789 bp longer than the short one, whereas medium and long forms differ in 1089 bp. Until now, there is no conclusive information about potential associations between promoter use and alternative polyadenylation sites. However, the type III isoform seems to be expressed in combination with all the three reported 3´ UTRs (Raynaud *et al.*, 2005b).

A potential association between the relative abundance of *CAST* isoforms and beef tenderness has been reported. Not only breed differences in beef tenderness but also among muscles of the same breed have been extensively documented (Rhee *et al.*, 2004; Calkins and Sullivan, 2007). Therefore, we took muscle samples of three muscles: *infraspinatus* (more tender), *triceps brachii* and *semitendinosus* (tougher), from a *Bos taurus* breed (Angus) and a *Bos indicus* breed (Brahman) that is known to produce consistently tougher beef compared to European breeds.

The objective of this study was to analyze the presence of APA variants of *CAST* in samples of the muscles and breeds mentioned above.

For this purpose we used the method known as "Rapid Amplification of 3´-cDNA End" (3´ RACE) (Frohman *et al.*, 1988) and sequencing to detect and characterize transcripts that differ in their 3´ UTR length.

MATERIALS AND METHODS

Samples

Within 1 h after slaughter, muscle samples (2 g) were taken from *infraspinatus*, *triceps brachii* and *semitendinosus* of 2 Angus steers (364±17 kg final body live weight and 19 months of age on average) and 2 Brahman steers (408±12 kg final body live weight and 42 months of age on average) and stored in liquid nitrogen or at 4 ºC as needed. The steers were slaughtered at two local private abattoirs: *Carnes del Salado SA* (Castelli, Buenos Aires, Argentina) and *Don Rafael SRL* (Santo Tomé, Corrientes, Argentina) for Angus and Brahman respectively. The animals were slaughtered after a 24 h rest in paddocks without feed but with access to water, according to the Handbook of Procedures for Animal Welfare of the National Service for Animal Health (*Servicio Nacional de Sanidad Animal*, *SENASA*) of Argentina.

RNA isolation

Approximately 100 mg of muscle tissue pulverized in a small amount of liquid nitrogen with a cooled pestle were mixed in 1 mL of TRIzol reagent (Life Technologies Corporation, CA, USA) and homogenized with the help of a mixer (Velp Scientifica®, Usmate, MB, Italy). The supernatant (aqueous phase containing the RNA) of each homogenate was obtained according to the manufacturer's protocol. The aqueous phase was mixed with ethanol (Sigma-Aldrich, St Louis, MO, USA) and total RNA was purified with RNA Clean & Concentrator®-5 kit (Zymo Research, Irvine, CA, USA) as per the manufacturer's instructions. RNA quality and concentration were determined by the $OD_{260/280}$ value (>1.7) with a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and confirmed by electrophoresis in agarose gels (0.8%)

stained with GelRed® Nucleid Acid Gel Stain (Biotium, Fremont, CA, USA).

Reverse transcription -3´ rapid amplification of cDNA ends-PCR

The 3´ rapid amplification of cDNA ends or 3´ RACE (Frohman *et al.*, 1988) is widely used to isolate the cDNA of unknown 3´ flanking sequences. The 3´ RACE technique was used to specifically amplify the different polyadenylation sites of *CAST* transcripts (Figure 1).

The oligodT used to generate the cDNA included an adapter sequence that was complementary to the *CAST* antisense primer (Table 1 and Figure 1). Two µg of RNA were used to produce first strand cDNA using 200 U of M.MLV Reverse transcriptase enzyme (Promega, Madison, WT, USA), 40 U of Recombinant Rnasin (Promega, Madison, WT, USA), 5 mM of DTT (Promega, Madison, WT, USA), 2.5 uM of oligodT-adapter (Table 1) and 10 pmoles of dNTPs (Promega, Madison, WT, USA).

Two specific sense oligonucleotides (Cast-e28 and Cast-e30) (Table 1) were designed to ensure the amplification of 3´UTRs of different length, as described by Raynaud *et al.* (2005b) and also predicted with Poly (A) Signal Miner software (Liu *et al.*, 2003).

Table 1. Oligonucleotide sequences used for cDNA synthesis (3´ RACE-PCR).

Name	Sequence (5´-3´)
Adapter-oligodT[1]	CCCGTCGACATGTACCAGTCCAAGCTTA
	GCGGCCGCATAGTTTTTTTTTTTTTTTT
Cast antisense	GGGCAGCTGTACATGGTCAGG
Cast-e28 (sense)	TTCCAGTGCCGAAGCACCTAGG
Cast-e30 (sense)	AACTAGGGAGGGTCTGATATCCGAC

[1]The underlined portion of the Adapter-oligodT sequence corresponds to the antisense primer sequence that was used in 3´ RACE-PCR.

The different end-point PCR reactions were performed with 3µL of cDNA, 10 pmoles of antisense oligonucleotide (Cast-antisense) which hybridizes to the adapter region of the adapter-oligodT (Table 1), 10 pmoles of the corresponding sense primer (Cast-e28 or Cast-e30), 2 U of Platinum Taq Polymerase (Invitrogen, São Paulo, Brazil), 2mM of Cl2Mg and 10 pmoles of dNTPs. Cycling conditions were 94 °C 5 min, 35 cycles of 94 °C for 1 min, 60 °C for 30 sec and 72 °C for 1 min; followed by a final 2 min extension at 72 °C. Since there was no certainty about any preferences in polyA sites among muscles or breeds, all the obtained amplicons were considered. However, special attention received the amplicons of approximately 300 bp and 1100 bp (primer Cast-e28) and 1055 bp (primer Cast-e30) which corresponds to previously described APA variants (Raynaud *et al.*, 2005b).

Amplified PCR products were observed by 1.5% agarose gel electrophoresis with GelRed® Nucleid Acid Gel Stain (Biotium, Fremont, CA, USA). Selected bands were eluted with the PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Carlsbad, CA, USA) and cloned in the pGEM-T-easy system (Promega, Madison, WT, USA) and E*scherichia coli* DH5alfa competent cells according to the manufacturer´s protocol. Forward and reverse sequences (using Sp6 and T7 primers) were generated from each cloned amplicon in an Applied Biosystems 3100 DNA Sequencer. These sequences were then comparatively analyzed by BLASTn and aligned to the reference genomic sequence of calpastatin (71,657 bp, Genebank accession AH014526.2) for the identification of the different polyadenylation sites.

RESULTS

The presence of alternatively polyadenylated calpastatin transcripts in three bovine muscles (*infraspinatus*, *triceps brachii* and *semitendinosus*) from two cattle breeds (Angus and Brahman) was analyzed.

The 3´RACE-PCR system was designed in order to obtain amplicons of approximately 350 bp and 1200 bp for short and medium transcripts with the Cast-e28 oligonucleotide; and 1100 bp for the long polyadenylated transcript using with the Cast-e30 oligonucleotide. In the PCR that included the forward primer Cast-e28, four intense bands (approximately 350, 400, 800 and 1200 bp) were observed on the agarose gel, whereas in sample no. 6 a product of approximately 1300 bp was also amplified (Figure 2A). Figure 2B shows two intense bands (800 and 1100 bp) obtained by PCR amplification with forward primer Cast-e30. All these bands were eluted, cloned and sequenced to confirm their identity. Positive clones of 347, 390, 1108 and 1142 bp were obtained (Figure 1). Sequencing confirmed the existence of three expected polyadenylation sites at positions 69783, 70573 and 71657 of the *CAST* reference sequence (GenBank accesion AH014526.2) corresponding to short (347 bp) and medium (1142 bp) polyadenylated transcripts amplified with Cast-e28; and long (1108 bp) polyadenylated transcripts amplified with Cast-e30 (Figure 3). These results are in agreement with the APA sites reported by Cong *et al.* (1998), Raynaud *et al.* (2005b) and Natrass *et al.* (2014). Interestingly, PCR performed with Cast-e28 produced a novel APA site that was present in the three muscles of both breeds.

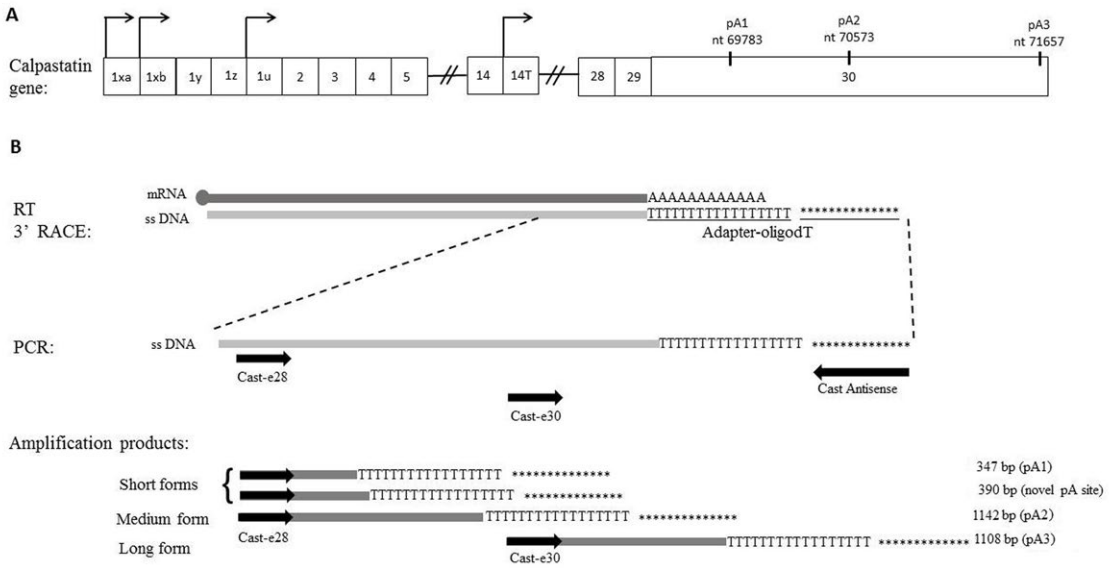

Figure 1. A. Schematic representation of calpastatin gene showing the four transcription start sites, exons and previously described polyadenylation sites (indicated as pA1, pA2 and pA3). **B.** Scheme of reverse transcription 3´RACE-PCR technique with the four amplification products obtained. "********" corresponds to sequence GATACGCCGCGATTCGAACCTGACCATGTACAGCTGCCC. *Cast* antisense primer is complementary to the adapter-oligodT. The PCR products are aligned with their corresponding forward primer (Cast-e28 or Cast-e30).

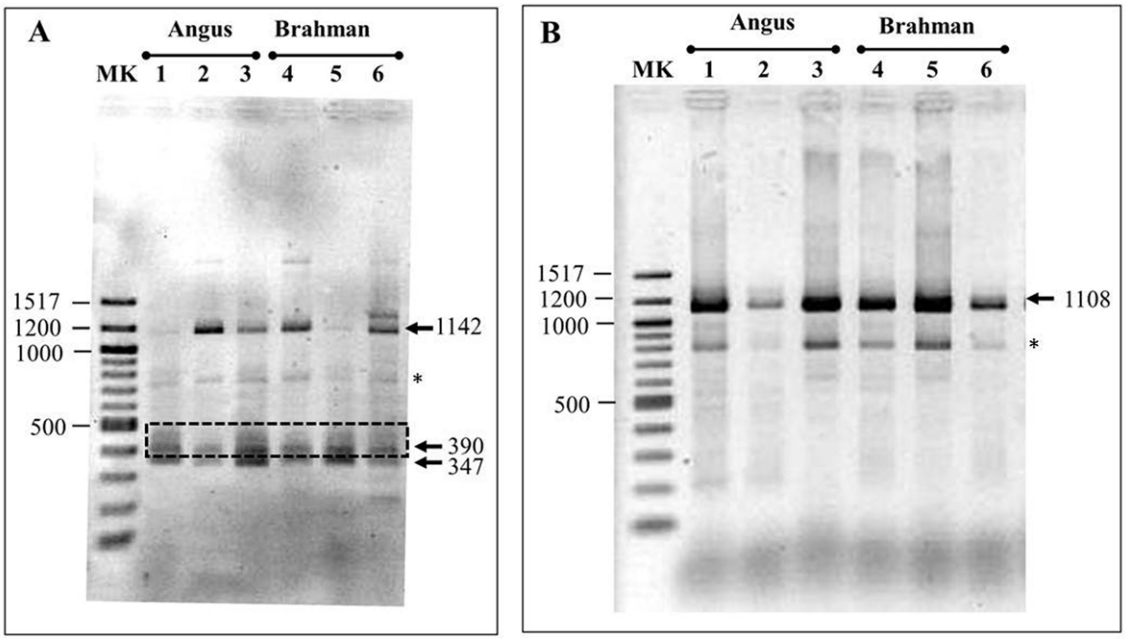

Figure 2. 1% agarose gel electrophoresis showing alternatively polyadenylated *CAST* transcripts amplified by 3´ RACE-PCR, using forward primers Cast-e28 (**A**) or Cast-e30 (**B**). Staining was performed with GelRed® Nucleid Acid Gel Stain (Biotium, Fremont, CA, USA). Each line was loaded with 10 µl of the PCR.
Lanes 1 and 4: *Infraspinatus*, 2 and 5: *Triceps brachii*, 3 and 6: *Semitendinosus*. MK: Quick-Load 100 bp DNA ladder (New England BioLabs, Hitchin, UK). Spurious fragments are indicated with a star (*). The amplicon corresponding to a novel pA site is indicated with a box.

This APA site is located in position 69817 of the reference sequence AH014526.2 (Figure 3) and corresponds to a 390 bp amplicon (Figure 2). Sequencing determined that the 800 bp (Figure 2A and B) and 1300 bp (Figure 2A, lane 6) bands corresponded to a spurious amplicon. The expression of the four different polyadenylated mRNA isoforms was confirmed in the 12 samples included in this study (Figure 2) and in all the samples that were analyzed for the implementation of the methodology (data not shown).

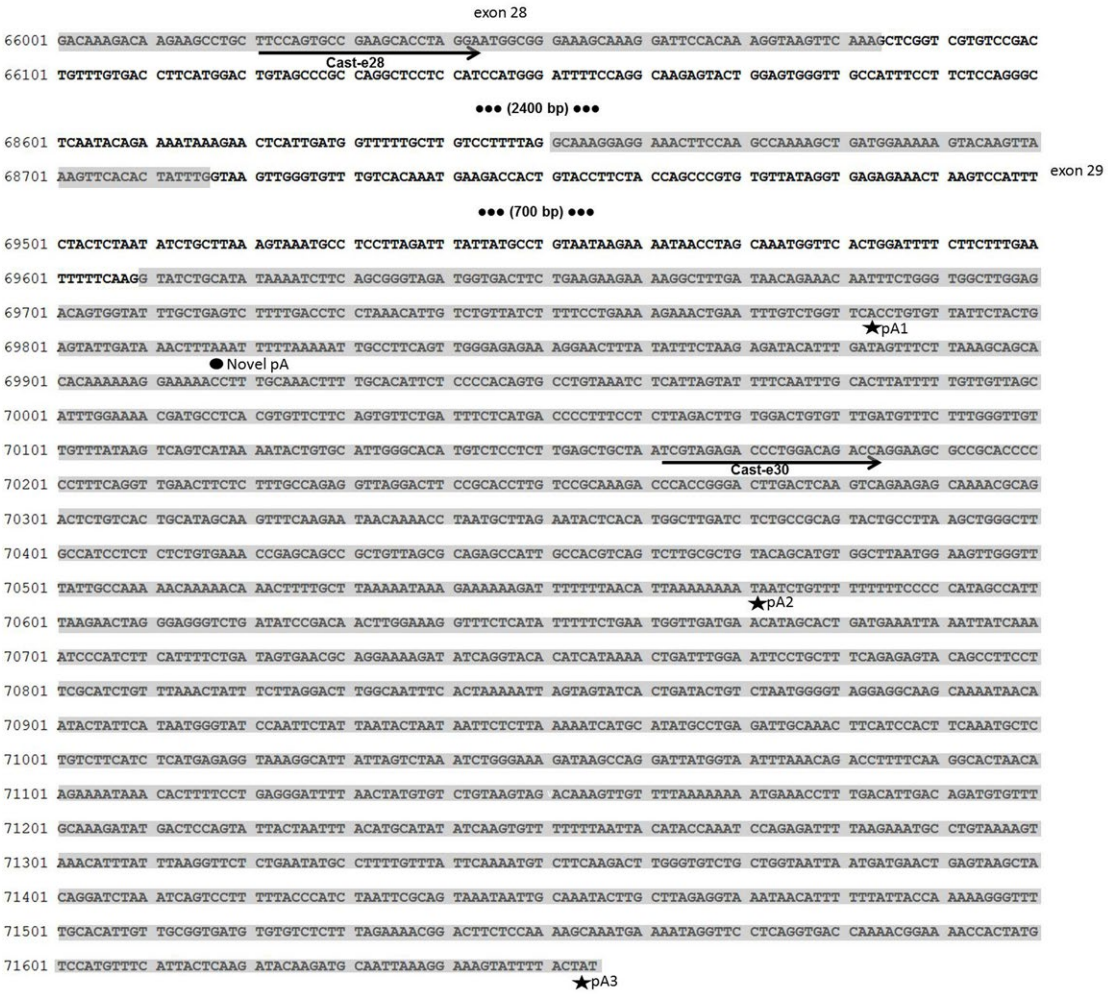

Figure 3. Sequence of the distal region of the calpastatin gene (Exons 28 to 30). Numbers on the left correspond to coordinates of reference sequence AH014526.2. Exons 28, 29 and 30 are shadowed. Non relevant parts of intron were removed and its base pairs annotated between brackets. Primers Cast-e28 and Cast-e30 are indicated with arrows. Known polyadenylation signals (pA1, pA2 and pA3) are indicated with a star (★); a circle (●) indicates a novel polyadenylation site.

DISCUSSION

There is still little information about the expression of *CAST* isoforms with different polyadenylation sites in different muscles or breeds, and its potential effects on beef quality traits. Nattrass *et al.* (2014) quantified two polyadenylation variants of *CAST* (those designated here as short and long, respectively) in the *longissimus lumborum* muscle of Angus and Brahman steers. The steers had been genotyped for the *CAST*:c.2832 A>G SNP, one of the first genetic markers for beef tenderness to be commercially available (Barendse, 2002). The findings of that study showed that a lower concentration of mRNA terminating at the proximal site (short) was significantly associated with the favorable allele for beef tenderness (A). These results supported the conclusion that *CAST*:c.2832 A>G SNP may be in linkage disequilibrium with regulatory sequences which have a role in the post-transcriptional processing of *CAST* transcripts, leading to reduced levels of calpastatin protein in muscles of individuals carrying the favorable allele. No association between *CAST*:c.2832 A>G SNP and polyadenylated forms were found in our samples since all the samples tested were homozygous for the A allele (data not shown).

A general correlation between the level of gene expression and the relative abundance of 3´ UTR isoforms have been reported (Ji *et al.*, 2011). The correspondence between APA and gene expression may be the consequence of the coupled usage of alternative promoters and polyA sites, previously reported for some genes (Costessi *et al.*, 2006; Winter *et al.*, 2007). Since mRNAs with short 3´UTRs are generally more stable due to avoidance of destabilizing elements binding to that region (Mayr and Bartel, 2009) and the escape from cellular mechanisms degrading long 3´UTRs (Hogg

and Goff, 2010), a comparatively higher expression of short 3´UTR isoforms would lead to a higher steady concentration of mRNA, and the opposite would also hold true.

The results reported here did not allow us to establish a connection between alternative polyadenylation of *CAST* and calpastatin activity. However, they show that alternative polyadenylation, probably coupled with the usage of alternative promoters, adds more complexity to the analysis of beef tenderization both between breeds and between muscles within a breed. According to Raynaud *et al.* (2005b), the type III isoform is the most abundantly expressed transcript in muscle, and it would also present polyadenylation variants.

Three known APA transcripts and a novel isoform of the bovine *CAST* gene were identified in three muscles that differ in tenderness, of two cattle breeds with known variation in calpastatin activity. Thus, variability in beef tenderness does not seem to be simply due to the presence or absence of a given APA form. Moreover, the biological significance and implications for muscle physiology of at least four alternative polyA sites are not well understood.

New research would be needed for the relative quantification of each isoform and the evaluation of their effects on calpastatin activity and ultimately, on beef tenderness. All known APA isoforms should be comparatively quantified in order to get a better understanding of the contribution of *CAST* expression to the variability in beef tenderness, both among breeds and muscles within a breed. The knowledge of the new isoform would help in the design of qPCR experiments and reinforces the concept that a complex gene such as *CAST* should be also evaluated through full length RNA sequencing to detect the potential associations between APA, alternative splicing and alternative promoter selection.

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