Identification and expression pattern of cationic amino acid transporter-1 mRNA in small intestinal epithelia of Angus steers at four production stages

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ABSTRACT: Although dietary supplementation of cationic AA (CAA), especially L-Lys, is known to be essential for optimal growth of beef cattle, the proteins responsible for absorption of CAA by bovine intestinal epithelia have not been described. Cationic AA transporter-1 (CAT-1) is a major intestinal CAA transporter, demonstrating a high-affinity (μM) transport activity for L-Lys in other mammals, and is widely expressed by small intestinal epithelia of nonruminants, but neither sequence nor expression pattern data exist for CAT-1 in cattle. Therefore, the goal of this research was to compare the relative expression (putative) of CAT-1 mRNA by duodenal, jejunal, or ileal small intestinal epithelia across and within commercially relevant beef cattle production and development stages. Twenty-four Angus steers were assigned randomly (n = 6) to 1 of 4 treatments (suckling, weanling, growing, and finishing) after all steers were born. Duodenal, jejunal, and ileal epithelia were scraped, and total RNA was extracted after the steers were killed at 32, 184, 248, or 423 d of age. Average daily gains of the steers did not differ (1.09 ± 0.05 kg/d) among stages, whereas the small intestinal length relative to BW decreased (P < 0.01) with steer development. Using standard reverse transcription-PCR cloning techniques, we generated a partial-length bovine CAT-1 complementary DNA (695 bp; GenBank accession no. DQ399522) from jejunal mRNA samples, which possessed 89 and 87% identities to pig and human CAT-1 orthologs, respectively. On the basis of this bovine-specific genetic data, a real-time PCR-based assay of reverse-transcribed mRNA was developed and used to measure relative changes in bovine CAT-1 mRNA abundance in intestinal epithelia as steers developed. The CAT-1 mRNA was expressed by the duodenum, jejunum, and ileum of all 4 production stages. In contrast to expression by duodenal or ileal epithelium, jejunal expression of CAT-1 mRNA by growing steers was greater (P = 0.005) than that by suckling, weanling, or finishing steers. In terms of the expression of CAT-1 mRNA within production stage, jejunal expression was greater (P = 0.002) than that by duodenum or ileum for growing steers. In contrast, no intestinal site difference was found for suckling, weanling, or finishing steers. These data indicate that previously reported Na+-independent uptake of L-Lys by jejunal and ileal epithelia likely occurred by CAT-1, and that the potential capacity for CAT-1-mediated uptake of CAA for beef steers may be greatest for the “growing” phenotype.

Key words: amino acid transport, bovine, cationic amino acid transporter-1, regulated gene expression, SLC7A1, small intestine

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INTRODUCTION

Cationic AA (CAA) are considered essential for optimal growth of beef cattle, with L-Lys being the first-limiting AA when a diet high in corn is fed (Titgemeyer et al., 1988; Merchen and Titgemeyer, 1992) and the second-limiting AA when microbes serve as the only protein supply (Richardson and Hatfield, 1978). Although bypass proteins (which have a relatively high L-Lys content) are typically fed as a source of supplemental L-Lys, very little is known about the transport proteins expressed by the small intestinal epithelia that are responsible for absorbing free CAA from luminal digesta (Moe et al., 1987; Matthews, 2000).

In mammals, 4 CAA transport system activities have been identified (y⁺, y⁺L, b₀⁺, B₀⁺). Of these, system y⁺ activity (Na⁺-independent uptake of L-Lys, L-Arg, L-Ornithine, and, to a lesser degree, L-His) is reported to be expressed along the entire small intestine on both
Table 1. Composition (% of DM) of diets consumed by developing Angus steers

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Suckling1</th>
<th>Weanling2</th>
<th>Growing</th>
<th>Finishing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suckled milk</td>
<td>100</td>
<td>26.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pasture</td>
<td>—</td>
<td>74.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Corn silage</td>
<td>—</td>
<td>—</td>
<td>89.0</td>
<td>35.7</td>
</tr>
<tr>
<td>Cracked corn</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>44.4</td>
</tr>
<tr>
<td>Alfalfa haylage</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>10.0</td>
</tr>
<tr>
<td>Supplement3</td>
<td>—</td>
<td>—</td>
<td>11.0</td>
<td>9.9</td>
</tr>
</tbody>
</table>

1From 12 to 24 h after birth, all steers were with their dams on a mixed pasture (50% orchardgrass, 25% fescue, and 25% clover) until slaughter at 32 ± 1 d (for the suckling group) or 184 ± 1 d (for the weanling group) of age. Water and mineral mix were available at all times to calves and dams. The growing and finishing groups were weaned and removed from the pasture at 180 ± 5 d of age, housed in a pen, and fed a high-stress adaptation diet for 7 d. The growing diet was fed to both groups for 31 to 33 d. The growing group was then slaughtered (248 ± 2 d), whereas the finishing steers were fed the finishing diet for 175 d before slaughter (423 ± 3 d).

2The ingredient composition for the suckling and weanling diets was estimated based on the values reported by Loy et al. (2002), Gelvin et al. (2004), and Reed et al. (2006).

3Supplement (on a DM basis) consisted of 83.79% soybean meal, 4.81% ground corn, 1.78% dicalcium phosphate, 6.66% limestone, 2.73% trace mineralized salt (98.5% NaCl, 0.35% Zn, 0.50% Mn, 330 mg/kg Cu, 70 mg/kg I, 50 mg/kg Co, and 90 mg/kg Se), and 0.23% vitamin premix (vitamin A 8,800 IU/g, vitamin D 1,760 IU/g, and vitamin E 1.1 IU/g).

Animal Trial Procedures

Care of Animals. Research protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Twenty-four purebred Angus cow-bull calf pairs were obtained from the University of Kentucky Agricultural Research Center Beef Unit. Calves were weighed and castrated from 12 to 24 h after birth, and vitamins A and D (1.0 mL; Sparhawk Labor Inc., Shawnee Mission, KS) and E with Se (0.5 mL; MU-SE, Schering-Plough Animal Health, Union, NJ) were administered. After all calves were born, steers were assigned randomly to 1 of 4 treatment groups, which were the suckling, weanling, growing, and finishing production stages (n = 6). All cow-calf pairs were maintained on a mixed pasture consisting of approximately 50% orchardgrass, 25% endophyte-free fescue, and 25% clover. All steers had ad libitum access to water and a mineral mix throughout the trial. Calves had unrestricted access to their dams, and the cow-calf pairs were observed daily for any signs of sickness. At approximately 84 d of age, the calves were vaccinated with Clostri Shield 7 Way and Vira Shield 5 plus Somnus (Grand Laboratories, Larchwood, IA), except for those in the suckling treatment group.

The compositions of the diets for the 4 production stages are presented in Table 1. Suckling (suckling milk) and weanling (grazing grass and suckling milk) treatment calves remained with their dams until slaughter at 32 ± 1 and 184 ± 1 d of age, respectively, whereas growing (fed a corn silage diet) and finishing (fed a high-concentrate corn diet) treatment calves were weaned as a group in a pen at 180 ± 5 d of age. The weaned calves were fed a high-stress adaptation diet consisting of (as-fed basis) 34.3% cracked corn, 15% cottonseed hulls, 15% whole oats, 16% soybean meal, 5% cane molasses, 4% dicalcium phosphate, 6% limestone, 3% trace mineralized salt (94% salt, 5.50 kg of Zn, 9.28 g/kg of Fe, 4.79 g/kg of Mn, 1.84 kg of Cu, 1.15 mg/kg of I, 0.18 mg/kg of Se, 0.65 mg/kg of Co; Burkmann Feeds, Danville, KY), 0.3% vitamin A (8,811 IU/g), D (1,762 IU/g), and E (11 IU/g) premix (ADM Alliance Nutrition, Quincy, IL), 1.2% decoquinate premix (6% decoquinate; Deccox, Alpharma Inc., Bridgewater, NJ), and 0.2% chlorotetracycline premix (110 g of chlorotetracycline/kg, Auromycin 50, Alpharma Inc.). After 7 d, these steers were moved to individual 3 × 3.7 m pens in an environmentally controlled room (21°C) with 16-h light:8-h dark cycles. For the first 30 d in the room, the steers were fed enough of a fescue hay (76%) plus soybean hull (23%) diet to maintain BW and were allowed to adapt to the individual pens. After the adaptation period, steers of both groups were fed enough growing diet (Table 1) to gain BW at 1 kg/d (NRC, 2000). After 31 to 33 d of feeding, growing treatment steers were slaughtered (at 248 ± 2 d of age),
whereas finishing treatment steers were moved and housed as 2 groups of 3 steers in 2 open-air, 8 × 30 m pens. Steers were fed enough of a high-concentrate, corn-based diet (finishing diet; Table 1) to gain BW at 1 kg/d for 175 ± 5 d. Fourteen days before slaughter at 423 ± 3 d of age, finishing calves were housed individually to facilitate determination of individual feed intake.

**Determination of Rates of Gain.** Body weights at birth and slaughter were recorded for all calves or steers. Suckling treatment calves were weighed additionally at 14 d of age to allow for a 3-point determination of growth. Steers in the weaning group were weighed every 28 d until slaughter and at 21 d before slaughter, whereas steers in the growing and finishing groups were weighed every 28 d until weaning. Once housed in the individual pens, growing and finishing steers were weighed weekly to facilitate adjustment of feed intake to meet the targeted ADG (1 kg/d). Once the growing period was over, the finishing steers were weighed again every 28 d. After housing finishing steers in individual pens, they were weighed every 7 d until slaughter.

**Slaughter and Tissue Collection.** Suckling treatment steers were anesthetized by intrajugular administration of pentobarbital (80 mg/kg of BW) and then exsanguinated during tissue removal. Steers of all other treatment groups were stunned with a captive-bolt pistol and exsanguinated. Serially, the liver, kidney, small intestine, and pancreas were removed. The total length of the small intestine (pyloric valve to ileal-cecal junction) was determined by looping the intestine across a board that was fitted with metal pegs at 2-m increments. Looping of the small intestine was performed without tension to minimize stretching, and the board was kept wet to minimize tissue shrinkage.

The sites and protocol of tissue sampling for RNA extraction were described previously by Howell et al. (2001). More specifically, 1-m sections of the duodenum (0.5 to 1.5 m caudal to the pyloric junction), jejunum (middle of the first half of the nonduodenal small intestine), and ileum (middle of the second half of the nonduodenal small intestine) were removed. Each section was bisected, inverted, and rinsed with cold (4°C) physiological saline (0.9% NaCl). One half of each intestinal section was scraped to collect the epithelia (Matthews et al., 1996) and the other half was frozen in liquid N2.

**Total RNA Extraction and Reverse Transcription-PCR**

**Total RNA Extraction.** Total RNA was extracted from approximately 1 g of scraped epithelia of duodenal, jejunal, and ileal samples from each steer. The RNA extraction was performed by using TRIzol reagent, following the instructions of the manufacturer (Invitrogen, Carlsbad, CA). To obtain RNA for reverse transcription (RT) reactions, 1 g of epithelial tissue was homogenized in 5 mL of TRIzol. After total RNA was recovered, the RNA pellet was washed gently with 75% ethanol (0.5 mL) and centrifuged at 7,500 × g at 4°C for 5 min. The cleaned RNA was then suspended in RNase-free distilled H2O and stored at −80°C. The quality of the total RNA samples was determined by electrophoresis of RNA solutions on agarose gels and visualization of the integrity of the 18S and 28S RNA bands. The concentration, purity, and amount of total RNA were determined by UV spectrophotometry. With these standard procedures, nearly all RNA samples used for the assay were of high quality and purity (260/280 absorbance >1.7).

**RT-PCR.** For partial-length cloning of bovine CAT-1, the RT-PCR was performed by using a 2-step protocol designed in accordance with the manufacturer’s guidelines (Invitrogen). For the first step, 5 µg of total RNA was reverse-transcribed to complementary DNA (cDNA) by using a SuperScript III RT, Oligo(dT)20 primer, Random Hexamer, and deoxy nucleotide 5′-triphosphate (dNTP) mix. For the second step, all of the PCR reactions were performed in a Hybaid MultiBlock PCR System (Thermo Electron Corporation, Waltham, MA). Each 50-µL reaction mixture contained 4.0 µL of RT product (>200 ng) as DNA template, 0.8 µM each forward and reverse primer (Table 2), 1.5 U of Platinum Taq DNA Polymerase, 4.0 mM MgCl2, and 0.2 mM of each dNTP (all from Invitrogen). The thermal cycle program for the PCR consisted of 1 cycle at 94°C (5 min), 38 cycles at 94°C (0.5 min), 62.5°C (0.5 min), and 72°C (1.0 min), 1 cycle at 72°C (10 min), and 1 cycle at 4°C. The forward and reverse primers were designed based on a pig CAT-1 cDNA sequence (GenBank accession no. NM_001012613; Table 2 and Figure 1).

**Cloning and Sequence Analysis of a Partial-Length Bovine CAT-1 cDNA**

**Cloning.** The putative CAT-1 PCR products were purified from the PCR reaction mixture by using a PureLink Quick Gel Extraction Kit (Invitrogen) and then amplified by using the TA Cloning Kit (Invitrogen) in accordance with the manufacturer’s instructions. Briefly, the PCR product was inserted into pCR II plasmid vector by ligation with T4 DNA Ligase overnight at 14°C; the resulting construct was then transfected into One ShotINVαF′ Chemically Competent Escherichia coli cells. The transfected E. coli cells were grown on Luria-Bertani agar plates containing ampicillin (at 100 µg/mL) and X-Gal (40 µL of a 40 mg/mL solution). After incubation of the plates at 37°C overnight, 10 to 20 white colonies were selected for further culture in 3 to 4 mL of Luria-Bertani-ampicillin (100 µg/mL) broth overnight at 37°C with vigorous shaking (∼250 to 300 rpm/min). The plasmids were then isolated from the E. coli cells by using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI) according to the manufacturer’s instructions. Recovered plasmids were subjected to restriction analysis by using endonucleases EcoRI and NcoI. The cDNA yielding the appropriate restriction patterns were sent to the DNA Se-
Bioinformatic Analysis of RT-PCR Products. The sequences of the molecular cloning products were compared with the template sequences for validation purposes by using the National Center for Biotechnology Information (NCBI) BLASTN computer program (version 2.2.12; www.ncbi.nlm.nih.gov/BLAST; last accessed Nov. 12, 2007). Multiple alignment of bovine CAT-1 cDNA sequences with those of other animal species reported in the literature was performed by using the ClustalW computer program (version 1.83) at the European Molecular Biology Laboratory–European Bioinformatics Institute (EMBL-EBI) Web site (http://www.ebi.ac.uk/clustalw/index.html; last accessed Nov. 12, 2007). During the alignment work, the default parameters with the program were used.

Real-Time RT-PCR Quantification of Bovine CAT-1 mRNA

RT. For relative quantification of CAT-1 gene expression, a real-time RT-PCR analysis was performed by using a 2-step protocol designed according to the manufacturer’s guidelines (Applied Biosystems, 2004). Total RNA was treated with DNase I (amplification grade, Invitrogen) as described by the manufacturer. Briefly, 1 μg of total RNA was combined with 1 μL of 10X reaction buffer, 7 μL of diethylpyrocarbonate-treated H2O, and 1 μL of DNase I and incubated at room temperature for 15 min. One microliter of 25 mM EDTA was then added to the DNase-treated total RNA and incubated at 65°C for 10 min to stop the reaction. The entire volume of DNase-treated total RNA (approximately 11 μL) was then used in the RT reaction, as suggested by the manufacturer (Invitrogen). Briefly, a solution of Hexamers (50 ng/μL) and Oligo(dT)20 (50 μM) primers (1 μL each) was added to each reaction. The reaction mixture was incubated at 70°C for 10 min and then chilled on ice for 1 min. Afterward, a solution containing 2 μL of RT buffer (10X), 2 μL of dithiothreitol (0.1 M), 4 μL of dNTP (10 mM each), 4 μL of MgCl2 (25 mM), and 1 μL of RNase Out (Invitrogen) was added to the reaction. After incubation at 37°C for 2 min, the reaction was incubated with SuperScript II reverse transcriptase (1 μL) at room temperature for 10 min, and then incubated at 37°C for 50 min. To stop the reaction, the reaction mixture was incubated at 70°C for 10 min and then chilled on ice. The resulting reaction products, which were cDNA, were then stored at −20°C until use in the real-time RT-PCR analysis of CAT-1 mRNA content.

Real-Time RT-PCR. The real-time PCR of RT products was performed by using an ABI Prism 7000 Sequence Detection System and a Custom TaqMan Primer and Probe set (Applied Biosystems, Foster City, CA), consisting of 2 unlabeled PCR primers and 1 TaqMan Minor Groove Binding probe with FAM, a reporter dye labeled at the 5′ end (Table 2). Components of a 25-μL PCR reaction were the Assays-by-Design Primer and Probe set (1.25 μL, Applied Biosystems), DNAse/RNase-free H2O (10.25 μL), cDNA template (1.0 μL), and TaqMan Universal PCR Master Mix-No AmpErase UNG (12.5 μL, Applied Biosystems). The PCR conditions used for the amplification and quantification were an initial denaturing stage (95°C for 10 min), followed by 40 cycles of 2 amplification stages of denaturing (95°C for 15 s) and annealing-extension (60°C for 1 min), with a melting curve program (60 to 95°C), a heating rate of 0.15°C/s, and continuous fluorescence measurements. Each tissue cDNA sample of each steer was performed in triplicate.

Relative quantitative measurement of CAT-1 mRNA expression was conducted by using a relative standard curve method and a serial dilution of RT cDNA. The standard curves for CAT-1 and 18S were constructed with a common basis cDNA sample, which was an aliquot pool of all the cDNA samples generated for quantification
Figure 1. Multiple alignment of cationic AA transporter-1 (CAT-1) gene sequences from the bovine, pig, human, mouse, and rat at the corresponding regions. The alignment was performed by using the ClustalW computer program (version 1.83) with default parameters. UK-bCAT-1 = the bovine CAT-1 sequence obtained from this study at the University of Kentucky (GenBank accession no. DQ399522); Pd-bCAT-1 = the newly released computer-predicted bovine CAT-1 sequence (GenBank accession no. XM_587329); sCAT-1 = the pig CAT-1 sequence (GenBank accession no. NM_001012613); hCAT-1 = the human CAT-1 sequence (GenBank accession no. NM_003045); mCAT-1 = the mouse CAT-1 sequence (GenBank accession no. NM_007513); and rCAT-1 = the rat CAT-1 sequence (GenBank accession no. NM_013111). The asterisk symbol (*) indicates identical bases across the 6 species. The percentage numbers at the end of the figure indicate the nucleotide sequence identities of each animal species compared with UK-bCAT-1. (Sequence continued on next page.)

(Applied Biosystems, 2004; Bustin, 2004; Larionov et al., 2005). The quantified levels of 18S ribosomal RNA were used as an endogenous control to normalize variations in mRNA inputs and RT reaction efficiencies (Bustin et al., 2005). Specifically, bovine small intestinal cDNA samples were serially diluted by 2.5-, 5-, 25-, 125-, 625-, 3,125-, 15,625-, and 78,125-fold, and the linear range for target quantification was established to ascertain an appropriate amount of cDNA to be used for the standard curve determination. The minimal threshold ($CT_{min}$) values detected by using these dilutions of cDNA were 34 to 38 for the target and control genes. Accordingly, the dilutions of the RT cDNA stocks for CAT-1 and 18S quantification were defined as being 5-
Figure 1 Continued. Multiple alignment of cationic AA transporter-1 (CAT-1) gene sequences from the bovine, pig, human, mouse, and rat at the corresponding regions. The alignment was performed by using the ClustalW computer program (version 1.83) with default parameters. UK-bCAT-1 = the bovine CAT-1 sequence obtained from this study at the University of Kentucky (GenBank accession no. DQ399522); Pd-bCAT-1 = the newly released computer-predicted bovine CAT-1 sequence (GenBank accession no. XM_587329); sCAT-1 = the pig CAT-1 sequence (GenBank accession no. NM_001012613); hCAT-1 = the human CAT-1 sequence (GenBank accession no. NM_003045); mCAT-1 = the mouse CAT-1 sequence (GenBank accession no. NM_007513); and rCAT-1 = the rat CAT-1 sequence (GenBank accession no. NM_013111). The asterisk symbol (*) indicates identical bases across the 6 species. The percentage numbers at the end of the figure indicate the nucleotide sequence identities of each animal species compared with UK-bCAT-1.

and 15,625-fold, respectively. The C\textsubscript{T} values for 18S mRNA across samples of this study were statistically analyzed to determine whether there was any treatment or tissue effect on 18S expression. For the control of system error during sample preparation, the relative CAT-1 RNA expression was normalized to that of 18S by calculating the ratios of CAT-1:18S relative mRNA quantities. These 18S-normalized ratios were then used for statistical analysis of CAT-1 mRNA expression.

Sequence Validation of Real-Time RT-PCR Products

To prepare the real-time RT-PCR products for sequencing, a PureLink Quick Gel Extraction Kit (In-
RESULTS AND DISCUSSION

Animal Performance

Average daily gain did not differ among treatment groups. As planned and expected, the birth BW for suckling, weanling, growing, and finishing treatment groups did not differ \( (P = 0.83) \), whereas the final BW at slaughter did \( (P < 0.001) \); Table 3). The final BW increased \( (P < 0.001) \) significantly from the suckling to weanling, weanling to growing, and growing to finishing stages. However, growth rates, measured either as overall ADG (calculated for the whole experiment period from birth to slaughter) or as final period ADG (calculated for the last 14 d before slaughter for suckling and the last 21 d before slaughter for the other treatment groups), did not differ \( (P = 0.37 \) and 0.21, respectively), with the values ranging from 1.0 to 1.2 kg/d.

The relative length of the small intestine decreased as steers developed. To evaluate the potential relationship between CAT-1 mRNA expression and tissue size, the potential treatment effects on small intestinal length and the relative length as a ratio to BW were evaluated (Table 3). The total absolute length of the small intestine increased \( (P < 0.05) \) from suckling to weanling steers and then stabilized, with the weanling, growing, and finishing steers having a small intestinal length that was 45, 48, and 52\% longer than suckling steers, respectively. In contrast, the relative small intestinal length (ratio to BW) was greatest \( (P < 0.05) \) for suckling steers, being 133, 154, and 367\% longer than weanling, finishing, and growing steers. Thus, the relative length of the small intestine decreased with steer development.

Generation of a Partial-Length Bovine CAT-1 cDNA

At the initiation of this project, no ruminant-specific CAT-1 cDNA sequences had been reported. Accordingly, the full-length pig CAT-1 cDNA sequence \((\text{NM}_001012613; \text{Cui} \text{et} \text{al.}, \text{2005})\) was used as a template for designing the PCR primers (Table 2) for a putative bovine CAT-1 mRNA. The pig CAT-1 cDNA sequence was chosen because it shares the highest identities (93\%, 108/115) with a reported expressed sequence tag (GenBank accession no. BE758285) from a bovine cDNA library (Smith et al., 2001), although there was only a fragment of 115 bp that shared these identities in a NCBI nucleotide-nucleotide BLAST search. The CAT-1 sequences of humans, mice, and rats (GenBank accession nos. \(\text{NM}_003045, \text{NM}_007513, \\text{and} \) \(\text{NM}_001012613\)) shared only 86 to 88\% identities (93\%, 108/115) with a reported expressed sequence tag (GenBank accession no. BE758285) from a bovine cDNA library (Smith et al., 2001), although there was only a fragment of 115 bp that shared these identities in a NCBI nucleotide-nucleotide BLAST search. The CAT-1 sequences of humans, mice, and rats (GenBank accession nos. \(\text{NM}_003045, \text{NM}_007513, \text{and} \) \(\text{NM}_013111\), respectively) shared only 86 to 88\% identities with this reported bovine expressed sequence tag, and also only the same 115-bp fragment shared these identities in the BLAST search.

Two single-gel-band RT-PCR products (approximately 700 bp) were generated from 2 jejunal cDNA samples of 1 suckling and 1 growing Angus steer. These
Table 3. Animal BW, ADG, and small intestine length of the developing Angus steers

<table>
<thead>
<tr>
<th>Item</th>
<th>Suckling</th>
<th>Weanling</th>
<th>Growing</th>
<th>Finishing</th>
<th>SEM2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth</td>
<td>37.4</td>
<td>38.5</td>
<td>36.0</td>
<td>37.5</td>
<td>1.9</td>
<td>0.83</td>
</tr>
<tr>
<td>Slaughter3</td>
<td>72.9a</td>
<td>251.3b</td>
<td>288.3c</td>
<td>505.4d</td>
<td>9.2</td>
<td>0.001</td>
</tr>
<tr>
<td>ADG, kg/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Birth to slaughter</td>
<td>1.1</td>
<td>1.2</td>
<td>1.0</td>
<td>1.1</td>
<td>0.06</td>
<td>0.37</td>
</tr>
<tr>
<td>Final period4</td>
<td>1.1</td>
<td>1.2</td>
<td>1.0</td>
<td>1.2</td>
<td>0.11</td>
<td>0.21</td>
</tr>
<tr>
<td>Small intestine length</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total, m</td>
<td>20.6a</td>
<td>29.8b</td>
<td>30.4b</td>
<td>31.3b</td>
<td>1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Relative, m/kg of BW</td>
<td>0.28a</td>
<td>0.12b</td>
<td>0.11b</td>
<td>0.06c</td>
<td>0.01</td>
<td>0.001</td>
</tr>
</tbody>
</table>

a–d Means within a row that lack a common superscript letter differ (P ≤ 0.05).
1Values presented are treatment means observed from the suckling, weanling, growing, and finishing groups.
2The SEM reported is the largest SEM. Sample sizes (n) are different for different parameters. For BW and ADG, n = 6. For small intestinal length, n = 5 for the suckling group, n = 6 for the weanling and growing groups, and n = 4 for the finishing group.
3Ages at slaughter for the suckling, weanling, growing, and finishing treatment groups were 32 ± 1 d, 184 ± 1 d, 248 ± 2 d, and 423 ± 3 d, respectively.
4Final period represents the final 14 d (suckling treatment group) and 21 d (weanling, growing, and finishing treatment groups) before slaughter.

2 products were then purified and cloned, and several clones were generated from each of 2 PCR products and then were preliminarily verified by an EcoRI and NcoI endonuclease digestion check (data not shown). At least 2 digestion-verified clones for each PCR product were chosen randomly and sequenced. The sequencing results showed that each of these digestion-verified clones had a PCR product insert that was 695 bp in length. Alignment of these 4 sequences with the NCBI BLAST engine (Tatiana and Thomas, 1999) showed 100% (695/695) identities among them. This putative jejunal CAT-1 sequence then was aligned (Figure 1) with the corresponding regions of the pig, human, mouse, rat, and a newly predicted bovine CAT-1 cDNA sequence (GenBank accession no. NM_001012613, NM_003045, NM_007513, NM_013111, and XM_587329, respectively), revealing shared identities of 89, 87, 84, 85, and 99%, respectively. Consequently, this partial-length bovine CAT-1 cDNA sequence now resides in GenBank with accession number DQ399522.

From this partial-length bovine CAT-1 cDNA sequence, the corresponding AA sequence was deduced, and then aligned to these AA sequences deduced from the aforementioned pig, human, mouse, and rat sequences and the predicted bovine CAT-1 cDNA sequence. This analysis revealed that the partial-length bovine CAT-1 AA sequence shared 97, 92, 91, 92, and 100% homology with the pig, human, mouse, and rat sequences and the predicted bovine CAT-1 AA sequence, respectively (data not shown).

Real-Time RT-PCR Methodology Validation

Validation of Real-Time RT-PCR Products. As shown in Figure 2, the sequence of the CAT-1 real-time PCR product had 100% (74/74 bp) identity with the corresponding region of the end-point PCR product obtained in this study (GenBank accession no. DQ399522), which was used as the template for real-time PCR primer and probe design (Table 2). The sequence of the 18S real-time PCR product had 96% (96/100 bp) identity with the corresponding region of the reported bovine sequence (GenBank accession no. DQ222453), which was used as the template for real-time PCR primer and probe design (Table 2 and Figure 2). This 18S product, however, had 100% (100/100 bp) identity with the corresponding region of another reported bovine 18S gene sequence (GenBank accession no. AF176811). The DQ222453 sequence was reported by a research group in China, whereas AF176811 was reported by a research group in Canada. Differences between cattle breeds or geographic locations may contribute to the difference between North American (DQ399522, AF176811) and Asian (DQ222453) bovine 18S sequences. Overall, these sequence results validated the real-time PCR reactions used in this study, for either the target gene or the endogenous control.

Validation of Endogenous Control Expression Levels. To establish the expression pattern for CAT-1 transporter in duodenal, jejunal, and ileal epithelia of Angus steers at different production or developmental stages, the 18S rRNA gene was selected as an expression control for the relative quantification of target gene expression in real-time RT-PCR analysis. The Ct values for 18S mRNA obtained from the real-time PCR analysis of all samples can be used to represent the quantity of 18S gene expression (Applied Biosystems, 2004). Although numerous publications have revealed that 18S rRNA is the most stable reference gene that can be used as an expression control without checking for a treatment effect, potential treatment effects on the expression levels of 18S rRNA were evaluated by comparing the Ct values for the 18S rRNA isolated from all small intestinal samples (Bustin et al., 2005).
Figure 2. Comparison of the cationic AA transporter-1 (CAT-1) and 18S real-time reverse transcription-PCR product sequences (Product) to the GenBank-reported CAT-1 (accession no. DQ399522) and 18S (accession no. DQ222453) gene sequences that were used in this study as templates for the primer and probe design. In addition, a comparison of the 18S product with another partial-length 18S sequence (accession no. AF176811) reported in GenBank is shown. Underlines indicate the positions of the forward and reverse primers, the gray highlights mark the positions of the FAM-labeled probes, and vertical lines indicate the matching of identical bases.

ANOVA analysis revealed that there were no production stage effects ($P = 0.89$), no small intestinal region effects ($P = 0.35$), and no stage $\times$ region interactions ($P = 0.72$; Table 4) identified. The 18S expression data were further analyzed by 1-way ANOVA to check whether there was any treatment effect at each individual treatment level, because CAT-1 mRNA expression data warranted a further analysis at each individual treatment level (as follows) and 18S data were to be used as normalizers. One-way ANOVA results (Table 4) revealed that 18S rRNA expression did not differ among the 4 production stages for every intestinal region ($P = 0.45$ to 0.82) and also did not differ among the 3 intestinal regions for every production stage ($P = 0.14$ to 0.52).

Effect of Production Stage on CAT-1 mRNA Expression

The transport of CAA and the expression of CAT-1 are known to be modulated by a variety of stimuli, including cell proliferation, growth factors, hormones, cytokines, and levels of substrates (Devés and Boyd, 1998; Hatzoglou et al., 2004). However, how physiological development or production stages affect the expression of CAT-1 in the small intestine of mammals, including ruminants, is not known (Pacha, 2000). To test the effect of 4 commercially relevant production stages on CAT-1 expression in Angus steers, the relative content of CAT-1 mRNA expressed by duodenal, jejunal, and ileal epithelia of suckling, weanling, growing, and finishing steers was analyzed. Cationic AA transporter-1 mRNA was expressed by duodenal, jejunal, and ileal epithelia at all production stages (Table 5). Two-way ANOVA analysis revealed that there was a significant interaction ($P = 0.03$) between production stage and small intestinal region. Consequently, 1-way ANOVA was further conducted to delineate the expression responses to production stage within each small intestinal region. No differences ($0.20 < P \leq 0.47$) in CAT-1 mRNA expression were observed among the production stages in either duodenal or ileal epithelial tissues (Table 5). In contrast, production stages affected ($P = 0.005$) jejunal expression of CAT-1, with that by growing steers being

<table>
<thead>
<tr>
<th>Table 4. Comparison of the expression levels of 18S rRNA isolated from the epithelial tissues at several regions of the small intestine collected from developing Angus steers$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
</tr>
<tr>
<td>Jejunum</td>
</tr>
<tr>
<td>Ileum</td>
</tr>
<tr>
<td>SEM$^2$</td>
</tr>
<tr>
<td>P-value$^3$</td>
</tr>
</tbody>
</table>

$^1$Expression levels of 18S ribosomal RNA are presented as mean threshold cycles observed in the real-time reverse transcription-PCR assay. Epithelial tissues of the duodenum, jejunum, and ileum were collected from suckling ($32 \pm 1$ d), weanling ($184 \pm 1$ d), growing ($248 \pm 2$ d), and finishing ($423 \pm 3$ d) Angus steers.

$^2$Sample sizes associated with 3 means in the weanling group for 3 tissue comparison were the same (i.e., $n = 6$), which gave rise to equal SEM for the 3 means. So is true in the finishing group (i.e., $n = 5$). In any other comparison group, unequal SEM were obtained because of unequal sample sizes associated with the 3 means (i.e., $n = 5$ and 6), in which case the largest SEM is presented.

$^3$P-values obtained for the main effect of production stage (row), tissue (column), and their interaction. Stage = production stage.
Table 5. Comparison of the relative expression levels of cationic AA transporter-1 (CAT-1) mRNA isolated from the epithelial tissues at several regions of the small intestine collected from developing Angus steers1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Suckling</th>
<th>Weanling</th>
<th>Growing</th>
<th>Finishing</th>
<th>SEM2</th>
<th>Stage</th>
<th>Stage × tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>0.26</td>
<td>0.45</td>
<td>0.30a</td>
<td>0.54</td>
<td>0.26</td>
<td>0.47</td>
<td>—</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.33a</td>
<td>0.31a</td>
<td>0.71a,b</td>
<td>0.45a</td>
<td>0.09</td>
<td>0.005</td>
<td>—</td>
</tr>
<tr>
<td>Ileum</td>
<td>0.67</td>
<td>0.24</td>
<td>0.30a</td>
<td>0.22</td>
<td>0.29</td>
<td>0.20</td>
<td>—</td>
</tr>
<tr>
<td>SEM2</td>
<td>0.29</td>
<td>0.14</td>
<td>0.09</td>
<td>0.26</td>
<td>—</td>
<td>—</td>
<td>0.03</td>
</tr>
<tr>
<td>P-value3</td>
<td>0.27</td>
<td>0.31</td>
<td>0.002</td>
<td>0.37</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Means within a row that lack a common superscript letter differ (P < 0.01).
Sample sizes associated with 3 means in the weaning group for 3 tissue comparison were the same (i.e., n = 6), which gave rise to equal SEM for the 3 means. So is true in the finishing group (i.e., n = 5). In any other comparison group, unequal SEM were obtained because of unequal sample sizes associated with the 3 means (i.e., n = 5 and 6), in which case the largest SEM is presented.
P-values obtained for the main effect of production stage (row), tissue (column), and their interaction.

Effect of Small Intestinal Epithelia on CAT-1 mRNA Expression

Cationic AA transporter-1 expression varies considerably across tissues and cell types (Hatzoglou et al., 2004). To understand the influence of small intestinal region on the expression of CAT-1 transporter, the content of CAT-1 mRNA relative to 18S mRNA was compared among duodenal, jejunal, and ileal epithelia within a production stage. Although CAT-1 mRNA was expressed in all 3 regions at each of 4 production stages, no difference (0.27 ≤ P ≤ 0.37) in terms of CAT-1 expression among 3 regions was observed for suckling, weanling, or finishing steers (Table 5). However, in growing steers the expression of CAT-1 by jejunal epithelia was approximately 120, 130, and 60% greater than those by suckling, weanling, and finishing steers, respectively. Given that CAT-1 mRNA encodes for CAT-1 protein, which possesses Na+ independent uptake capacity of CAA activity, our detection of CAT-1 mRNA in jejunal and ileal epithelia of the developing Angus steers is consistent with the observation of Na+ independent uptake capacity in L-Lys measured in brush border membrane vesicles isolated from jejunal and ileal epithelia of growing Holstein steers (Wilson and Webb, 1990).

We previously (Howell et al., 2003) found that expression of ovine transporters was affected by differential rates of growth. Therefore, in the current study, we purposefully formulated the growing and finishing diets to maintain a common ADG (i.e., approximately 1 kg/d) to match that achieved by the suckling and weanling treatment groups. The use of these diets to maintain a constant ADG was successful (Table 3). Accordingly, the increase in jejunal CAT-1 mRNA expression by growing steers found in this study was likely the result of steer developmental stage, not the difference in growth rate.

Production stage-dependent cationic amino acid transporter-1 expression

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In general, the absorption mechanisms in ruminants for the end-products of protein digestion appear to be similar to those described in nonruminants (Matthews, 2000; Krehbiel and Matthews, 2003). Likewise, the duodenum, jejenum, and ileum of small intestinal epithelium appear to have different capacities to absorb AA, with essential AA being preferentially absorbed over nonessential AA (Bergen, 1978). In contrast to nonruminants, which appear to possess the greatest absorption capacity in the jejunum, the ileal region of sheep and cattle has been identified as possessing the greatest potential for free AA absorption (Johns and Bergen, 1973; Phillips et al., 1979; Guerino and Baumrucker, 1987). In growing steers, however, this may not be the case for CAA because the Na+ independent uptake capacity for Lys by jejunal and ileal brush border membrane vesicles of growing steers does not appear to differ...
(Wilson and Webb, 1990). Furthermore, if CAT-1 mRNA is proportional to CAT-1-mediated system y+ uptake capacity (Hyatt et al., 1997; Fernandez et al., 2001), then the findings of this study (Table 5) indicate that the jejunum expresses the greatest capacity for Na+-independent CAA uptake in growing animals (as appears to be the case for nonruminants), whereas no difference exists along the small intestinal epithelia in suckling, weanling, and finishing steers. However, it is still unknown whether the cattle small intestinal epithelium also expresses another AA transporter, BAT1, that also possesses Na+-independent CAA uptake activity (Chairoungdua et al., 1999).

In summary, understanding how developmental stages and dietary substrates regulate system y+ transport of CAA in the ruminant small intestine should facilitate the rational design of dietary CAA supplements for different stages of beef production by matching dietary load to absorptive capacity. To this end, we generated a partial-length cDNA for a bovine CAT-1 mRNA ortholog and then used this sequence information to generate primers for real-time PCR analysis of bovine CAT-1 mRNA expression by small intestinal epithelia. Our data identify, for the first time, that the mRNA for CAT-1, a major mammalian intestinal CAA transporter, is expressed by the duodenal, jejunal, and ileal epithelia of Angus steers during 4 commercially relevant production stages (suckling, weanling, growing, and finishing). This finding of CAT-1 mRNA in cattle small intestinal epithelia is consistent with previously reported Na+-independent uptake of Lys by jejunal and ileal epithelia. We also found that more CAT-1 mRNA was expressed by the jejunal epithelium of growing steers than the jejunalum of other steers, and that, within growing steers, the jejenum expressed more CAT-1 mRNA than did the duodenal or ileal epithelia.

LITERATURE CITED


Production stage-dependent cationic amino acid transporter-1 expression


