Ruminal and abomasal starch hydrolysate infusions selectively decrease the expression of cationic amino acid transporter mRNA by small intestinal epithelia of forage-fed beef steers^{1,2}

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ABSTRACT

Although cationic amino acids (CAA) are considered essential to maximize optimal growth of cattle, transporters responsible for CAA absorption by bovine small intestinal epithelia have not been described. This study was conducted to test 2 hypotheses: 1) the duodenal, jejunal, and ileal epithelia of beef cattle differentially express 7 mRNA associated with 4 mammalian amino acid (AA) transport activities: y^+ (CAT1), $B^{0,+}$ (ATB^{0,+}), $b^{0,+}$ ($b^{0,+}$ AT and rBAT), and y^+L (y^+ LAT1, y^+ LAT2, and 4F2hc), and 2) the expression of these mRNA is responsive to small intestinal luminal supply of AA substrates (derived from ruminal microbes) or glucose-derived energy (from starch hydrolysate, SH), or both. Eighteen ruminally and abomasally catheterized Angus steers (body weight = 260 ± 17 kg) fed an alfalfa cube-based diet at $1.33 \times$ net energy for maintenance requirement were assigned to 3 treatments ($n = 6$): ruminal and abomasal water infusion (control); ruminal SH and abomasal water infusion; and ruminal water and abomasal SH infusion. The dosage of SH infusion amounted to 20% of metabolizable energy intake. After 14 or 16 d of infusion, steers were slaughtered, duodenal, jejunal, and ileal epithelia were harvested, and total RNA was extracted. The relative amounts of mRNA expressed by epithelia were quantified using real-time reverse transcription-PCR. All 7 mRNA species were expressed by the epithelium from each region, but their abundance differed among the regions. Specifically, duodenal expression of CAT1 and ATB^{0,+} mRNA was greater than jejunal or ileal expression; ileal expression of $b^{0,+}$ AT, rBAT, and y^+ LAT1 mRNA was greater than

jejunal or duodenal expression, whereas the expression of y^+ LAT2 and 4F2hc mRNA did not differ among the 3 epithelia. With regard to SH infusion effect, ruminal infusion down-regulated or tended to down-regulate the jejunal expression of CAT1, rBAT, y^+ LAT2, and 4F2hc mRNA. Abomasal infusion down-regulated the jejunal expression of y^+ LAT2 mRNA and tended to down-regulate the jejunal expression of 4F2hc mRNA. This study characterized the pattern of CAA transporter mRNA expressed by growing beef cattle fed an alfalfa-based diet. Moreover, this study demonstrated that increasing the luminal supply of microbe-derived AA (by ruminal supplementation of SH) results in a reduced capacity of apical and basolateral membrane to transport of CAA, whereas increasing luminal glucose supply (by abomasal supplementation of SH) reduces only the basolateral transport capacity, assuming that CAA transporter mRNA content represents functional capacity.

Key words: bovine, regulated gene expression, SLC3, SLC6, and SLC7

INTRODUCTION

Cationic AA (CAA; L-lysine, L-arginine, and L-histidine) are considered essential for the optimal growth of cattle, with lysine being first-limiting when a diet high in corn is fed (Titgemeyer et al., 1988; Merchen and Titgemeyer, 1992) and second-limiting when rumen microbes serve as the only protein supply (Richardson and Hatfield, 1978). However, little is known about the transporter proteins expressed by the small intestinal epithelia responsible for absorbing lysine and other free CAA from luminal digesta (Moe et al., 1987; Matthews, 2000) and whether they are sensitive to nutrient supply.

As for other mammals, the absorption of free AA from digesta by cattle occurs mainly in the small intestine (Bergen, 1978; Krehbiel and Matthews, 2003). Understanding how the small intestine regulates its capacity to absorb CAA is essential for beef nutritionists and producers to manipulate beef diets to achieve maximal, yet efficient, CAA absorption.

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In other mammals, 7 proteins (associated with 4 CAA transport system activities) are responsible for CAA absorption by the small intestinal epithelia (Devés and Boyd, 1998; Krehbiel and Matthews, 2003). These proteins are **CAT1** (system y^+), **ATB**^{0,+} (system B^{0,+}), **b**^{0,+}**AT** and **rBAT** (system b^{0,+}), and **y**⁺**LAT1**, **y**⁺**LAT2**, and **4F2hc** (system y^+L ; Devés and Boyd, 1998; Krehbiel and Matthews, 2003). The expression of CAT1 mRNA has been identified in the small intestinal epithelia of beef steers at 4 different developmental stages (Liao et al., 2008a), whereas expression of mRNA for the other 6 CAA transporter (**CAAT**) proteins comprising the 3 other CAA transport system activities has not. Moreover, the potential ability of increased AA substrate (in the form of microbial protein) or glucose (to the small intestine absorptive epithelia that express CAAT and absorb CAA) to regulate the expression of CAAT mRNA by small intestinal epithelia has not been evaluated.

Understanding the expression profile and the nutritional regulation of CAAT expression by small intestinal epithelia of beef steers will facilitate the matching of metabolizable AA supply with small intestinal absorption capacities. Previously, we reported (Liao et al., 2008b) that ruminal infusion of corn starch hydrolysate (**SH**) to growing steers increased duodenal expression of CNT3, ENT1, and ENT2, and ileal expression of CNT3 and ENT2 nucleoside transporter mRNA, whereas abomasal infusion only increased ileal expression of ENT2 mRNA. In this experiment, using the RNA extracted from these animals, we evaluated 2 hypotheses: 1) duodenal, jejunal, and ileal epithelia of beef steer express all 7 species of mRNA associated with 4 CAAT system activities, and 2) expression of these CAAT mRNA species are responsive to the luminal supply of AA (derived from rumen microbes), glucose (derived directly from SH), or both.

MATERIALS AND METHODS

Animal Trial Procedures

The description of animals used in this study, and their care, has been described previously and was approved by the University of Kentucky Institutional Animal Care and Use Committee (Liao et al., 2008b). Specifically, 18 Angus steers (BW = 260 ± 17 kg) were raised in the University of Kentucky Agricultural Research Center Beef Unit from February to August 2006. Because of facility and technical requirements, these steers were obtained in 3 staggered periods to minimize the differences in BW at the initiation of the animal trial. For each of the 3 staggered periods, a randomized complete block experimental design was employed.

Six steers in each period were blocked by BW (heavy vs. light weight). Within each of the 2 blocks, 3 steers surgically fitted with ruminal and abomasal infusion catheters (Walker and Harmon, 1995) were randomly assigned to 3 infusion treatments: 1) ruminal and abomasal infusion with water (control), 2) ruminal infusion with SH and abomasal infusion with water (ruminal infusion), and 3) ruminal infusion with water and abomasal infusion with SH (abomasal infusion). The dosage of SH infusion amounted to 20% of ME intake (approximately 800 g/d) based on individual animal BW determined at initiation of the SH infusion period. The equalized dosage (20% of ME intake) shared by ruminal and abomasal infusion was based on the assumption that essentially all of the ruminally infused SH would be fermented in the rumen (Walker and Harmon, 1995).

The basal diet fed to all steers was blended high-quality alfalfa-hay cubes (Tisdale Alfalfa Dehy Ltd., Tisdale, Saskatchewan, Canada) that contained 17.8% CP and 1.31 Mcal of NE_M/kg (DM basis), and was provided to steers at $1.33 \times$ ME requirement calculated as $0.077 \text{ Mcal/EBW}^{0.75}$ (where $\text{EBW}^{0.75}$ is metabolic BW based on empty BW, kg) of NE_M (NRC, 1996). The DM content of the alfalfa-hay cubes was 88.1%, and the OM, NDF, ADF, and acid detergent lignin (ADL) contents were 90.6, 44.4, 28.1, and 7.8% (DM basis), respectively. The trace mineral salt (92–96% NaCl) contained (mg/kg) Zn (5,500), Mn (4,790), Cu (1,835), Fe (9,275), I (115), Co (65), and Se (18) and was provided to steers at a dosage of 40 g/d. Steers also received 20 g/d of poloxolene (Phibro Animal Health, Ridgefield Park, NJ) to minimize the incidence of bloat. Steers were fed daily in 12 equally proportioned meals (once every 2 h) using automatic feeders (Ankom Co., Fairport, NJ), and had ad libitum access to fresh water throughout the trial. Steers were tethered individually in stalls (1.2 × 1.7 m) and housed in an environmentally controlled room (ambient temperature 20°C) with a 24-h light time.

The SH infusate used was a tap water solution of raw corn starch, partially hydrolyzed by a heat-stable α -amylase (Bauer et al., 1995). The SH was chosen over raw corn starch because the digestion characteristics of SH are similar to those of native starch yet it is more suspendable in solution and, therefore, facilitates pumping. Stock SH infusate solutions were prepared in 3 to 4 batches for each experimental period and stored at –20°C until use. Before infusion, the calculated amounts of stock solution were diluted with tap water to a final weight of 5.5 kg for each animal and infused over 22 h per day. During infusion, the homogeneity of the infusate solution was maintained by rapid continuous mixing of the solution on 6 individual stir-plates.

The SH solution or water (5.5 kg) was continuously infused at a rate of 250 mL/h to the animals to help maintain a steady-state condition of SH supply. The infusion was continued for 14 or 16 d before tissue sample collection.

Throughout the course of the animal trial, 2 steers were lost from the ruminal SH infusion group. One was due to factors unrelated to the infusion treatments, and the other stopped eating gradually during the late phase of the experimental period.

Animal Slaughter and Tissue Collection

After the 14- or 16-d SH or water infusion, 3 steers (i.e., 1 block) per day were transported to a USDA-approved slaughter facility for slaughter and tissue harvesting. Steers were killed by stunning with a captive-bolt pistol, followed by exsanguination. The small intestine was removed, and its total length (from pyloric valve to ileo-cecal junction) was determined by looping the intestine across a wet stationary board that was fitted with metal pegs at 2-m increments. The time elapsed between stunning and completion of small intestinal epithelia collection was approximately 35 min.

The sites and protocol for collection of small intestinal epithelial samples for total RNA preparation were as described previously by Howell et al. (2001, 2003). Briefly, 1-m sections of duodenum (0.5 to 1.5 m distal to the pyloric junction), jejunum (middle of the first half of nonduodenal small intestine), and ileum (middle of the second half of nonduodenal small intestine) were taken after removing the digesta. Each section was cut in half, inverted, and rinsed with ice-cold (4°C) physiological saline (0.9% NaCl), and then either scraped with a glass slide to collect the epithelia (Howell et al., 2001) or snap-frozen in liquid nitrogen for reserve (not analyzed for this study). Approximately 2 g of scraped epithelium was placed into 20 mL of TRIzol reagent (Invitrogen Corporation, Carlsbad, CA), homogenized immediately, and stored at -80°C.

RNA Extraction and Reverse Transcription

Total RNA Extraction and Purification. Total RNA was extracted from the frozen epithelial homogenate following the instructions for the TRIzol reagent (Invitrogen Corp.). After total RNA was recovered, a purification procedure was performed using RNeasy Mini Kit (Qiagen, Valencia, CA) to minimize genomic DNA contamination (Applied Biosystems, 2004) and enrich all the mRNA longer than 200 bp. Purified RNA was then eluted with 60 µL of RNase-free distilled H₂O and stored at -80°C. The integrity of the purified RNA was examined by gel electrophoresis using Agilent 2100

Bioanalyzer System (Agilent Technologies, Santa Clara, CA) at the University of Kentucky Microarray Core Facility. Visualization of the gel images and electrophoretograms showed that all RNA samples had high quality with an RNA integrity number >8.0 and a 28S/18S rRNA ratio >1.8. The purity and concentration of the purified RNA samples was analyzed by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), which revealed that all samples were of high purity with 260/280 absorbance ratios >2.0 and 260/230 absorbance ratios >1.75.

Reverse Transcription. Approximately 3 µg of total RNA was first treated with DNase I enzyme (amplification grade) in accordance with the manufacturer's instructions (Invitrogen). Briefly, a total RNA sample was combined with 1 µL of 10× reaction buffer, 1 µL of DNase I (1 U/µL), and diethyl pyrocarbonate-treated H₂O up to 10 µL, and incubated at room temperature for 15 min; then, 1 µL of 25 mM EDTA was added to stop the reaction by incubating at 65°C for 10 min. Then, the DNase-treated RNA samples were reverse transcribed to cDNA by using SuperScript III First-Strand Synthesis System in accordance with the manufacturer's instructions (Invitrogen). Briefly, a solution of hexamers (50 ng/µL) and oligo (dT)₂₀ primer (50 µM) mix (1 µL each) was added to one DNase-treated sample (7 µL in volume), incubated at 70°C for 10 min, and chilled on ice for 1 min. A solution containing 2 µL of reverse transcription (RT) buffer (10×), 2 µL of dithiothreitol (0.1 M), 4 µL of MgCl₂ (25 mM), 1 µL of dNTPs (10 mM each), and 1 µL of RNase Out were added to the reaction. After incubation at 37°C for 2 min, the reaction was incubated with 1 µL of reverse transcriptase at room temperature for 10 min, and then incubated at 50°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, cDNA, were stored at -20°C until used in the real-time PCR analysis.

Real-Time PCR

Before conducting real-time PCR with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), primer and probe sets for amplification of CAT1, ATB^{0,+}, b^{0,+}AT, rBAT, y⁺LAT1, y⁺LAT2, and 4F2hc cDNA were designed and manufactured using ABI Assays-by-Design Service (Applied Biosystems). Because no validated sequences for the 6 bovine CAAT-associated mRNA (except for CAT1) had been reported in the literature at the initiation of this project, their respective virtual sequences acquired from The Institute for Genomic Research (**TIGR**, <http://www.tigr.org>, Rockville, MD) and En-

Table 1. Primer and probe sets used for the real-time quantitative PCR analyses of cDNA derived from cationic amino acid transporter mRNA

Primer and probe ¹	Location (bp) on template	Sequence ²	Amplicon size (bp)
ATB ⁰⁺ (TIGR TC297793)			
Forward	831–856	5'-AGTCGGAGCAGCATTATTTAAAGGAA-3'	
Probe (rev.) ³	861–878	5'-FAM-ACCACCTTTCCAGAAGAC-3'	93
Reverse	899–923	5'-ACCAGGATGAGTAGGACAACATAGG-3'	
b ⁰⁺ AT (TIGR TC265966)			
Forward	179–197	5'-AGAGCAGCGAACCCTAAGAC-3'	
Probe (rev.)	211–225	5'-FAM-CAGGCCAGCTCCTT-3'	67
Reverse	226–245	5'-ATGATGCAGGTGCCACTGAA-3'	
rBAT (TIGR TC299989)			
Forward	755–778	5'-CATGACTGTAACCGTGAAAATGGT-3'	
Probe (fwd.)	791–809	5'-FAM-CCCAACAACCTGGTTAAGTG-3'	82
Reverse	815–836	5'-CAAAGTGCCAACTGGAGTTTCC-3'	
y ⁺ LAT1 (TIGR TC264913)			
Forward	707–729	5'-CATCGCAGGCATTGTTAGACTTG-3'	
Probe (fwd.)	731–747	5'-FAM-CCAGGGAGCCTCGACTC-3'	80
Reverse	762–786	5'-CCACTGAAAATGATGAACCCTCAAA-3'	
y ⁺ LAT2 (Ensembl ENSBTAT00000016074)			
Forward	498–513	5'-CCGTCTCCTCGCTGCA-3'	
Probe (fwd.)	515–531	5'-FAM-CCTGCATGTGTCTGCTG-3'	56
Reverse	532–553	5'-CATAGGCGCAGTTCACAAATGT-3'	
4F2hc (Ensembl ENSBTAT00000030071)			
Forward	1206–1230	5'-CCCAACACCTCAGCATCTATAACA-3'	
Probe (rev.)	1238–1255	5'-FAM-CTCTGGCCCTTCACAGTC-3'	77
Reverse	1263–1282	5'-AGGGAGAGGAGGGAGTTACG-3'	

¹The accession numbers of the sequences retrieved from the public databases (TIGR and Ensembl) and used as templates for designing primers and probes are shown in parentheses after the sequence name.

²FAM = 6-carboxy-fluorescein, a reporter dye used in the real-time PCR procedure.

³The custom TaqMan probes were supplied in either the reverse (rev.) or the forward (fwd.) orientation as indicated for each probe.

sembl (<http://www.ensembl.org/index.html>, European Bioinformatics Institute and Wellcome Trust Genome Campus Hinxton, Cambridgeshire, UK) public genomic databases were employed as templates for primer and probe design (Table 1). The primers and probe sets for CAT1 mRNA and 18S rRNA have been previously reported by us (Liao et al., 2008a). Each Assays-by-Design primer and probe set consists of 2 unlabeled PCR primers and one TaqMan minor groove binding probe labeled at the 5' end with a reporter dye, 6-carboxy-fluorescein (Table 1). To eliminate genomic DNA contamination, all primer and probe sets were designed to produce amplicons bridging exon-exon junctions (Ausubel et al., 2005; Cui et al., 2007).

Components of a 25-μL real-time PCR reaction were an Assays-by-Design primer and probe set (1.25 μL), TaqMan Universal PCR Master Mix-No AmpErase UNG (12.5 μL), cDNA template (1.0 to 2.0 μL), and DNase/RNase-free H₂O (9.25 to 10.25 μL). 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 for 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.

Development of mRNA Quantification Methodology

RT-PCR Product Validation. To establish mRNA relative quantification methodology, the real-time RT-PCR products were validated by DNA sequence verification. To prepare PCR products for sequence verification, a PureLink Quick Gel Extraction Kit (Invitrogen) was used to purify the PCR products from the reaction mixtures, which include unincorporated nucleotides, primers, and DNA polymerase. The real-time PCR mixture also included the 6-carboxy-fluorescein dye that was labeled to TaqMan probe by the manufacturer (Applied Biosystems). Approximately 250 μL of pooled PCR reaction mixtures were electrophoresed in a 1.2% agarose slab gel. A single cDNA band at the desired size was identified under a UV light, excised from the gel, placed into a sterile 1.5-mL polypropylene centrifuge tube, dissolved with the gel solubilization buffer, and then filtered through an extraction column. The column-bound cDNA was cleaned with the washing buffer, eluted with 25 to 40 μL of DNase/RNase-free H₂O, and concentration determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The cDNA were then sequenced using the fluorescent dideoxy-mediated chain termination method and a Perkin Elmer/Applied Biosystems automated DNA

Sequencer by the University of Florida DNA Sequencing Core Laboratory (Gainesville, FL). The resulting sequences were compared with the template sequences retrieved from TIGR and Ensembl genomic/nucleotide databases to confirm their identities.

Relative mRNA Quantification Methods. For quantification of the CAAT-associated mRNA abundance (expression levels), the relative quantitative real-time RT-PCR methodology that used a 2-step regimen was developed in accordance with ABI guidelines (Applied Biosystems, 2004). In the first step, all total RNA samples were reverse-transcribed to cDNA as described above for the RT reaction. In the second step, 7 relative standard curve methods were established for the 7 CAAT-associated cDNA respectively, and the 18S cDNA (reverse-transcribed from 18S rRNA) was selected as an endogenous control to normalize the variations in sample preparation, mRNA inputs, and RT efficiencies (Liao et al., 2008a). Specifically, a cDNA sample was serially diluted $2.5\times$, $5\times$, $25\times$, $125\times$, $625\times$, $3,125\times$, $15,625\times$, $78,125\times$, and $390,625\times$, and the linear range for target mRNA quantification was established to ascertain an appropriate amount of cDNA to be used for the standard curve method. For each cDNA sample the real-time PCR reactions (as described above) were conducted in triplicate to average out the potential pipetting, mixing, or plate setting-up errors. The minimal cycle threshold (C_T) values detected using these dilutions were around 21 to 37 and 22 to 23 for the 7-target and 18S cDNA, respectively. As a result, the optimal detection of CAAT and 18S cDNA was achieved by using 1:5 and 1:15,625 dilutions of the RT product stocks, respectively.

The potential tissue distribution and SH infusion treatment effects on the expression of 18S rRNA by small intestinal epithelia were evaluated by comparing the C_T values obtained from the real-time PCR reactions (Applied Biosystems, 2004). The relative quantities of CAAT-associated mRNA expression were normalized to the relative 18S quantities by calculating the CAAT:18S relative quantity ratios, and these 18S-normalized quantity ratios were used for mRNA tissue distribution pattern analysis (i.e., the expression levels associated with non-SH infused duodenal, jejunal, ileal epithelia). For SH infusion treatment effect on CAAT-associated mRNA expression, the mean of the 18S-normalized ratios from the control animals (i.e., without SH infusion) was designated as the calibrator. Then, the 18S-normalized ratios of the ruminal, abomasal SH infusion, and control animals were divided by the calibrator. The C_T values for 18S rRNA quantities, the normalized values for mRNA tissue distribution pattern, and the calibrated values for SH infusion effect were all subjected to statistical analysis.

Statistical Analysis

Statistical analyses were performed as described for this animal model (Liao et al., 2008b). Specifically, the effects of small intestine epithelial region (duodenal, jejunal, and ileal), and the effects of SH infusion (control, ruminal, or abomasal) treatment on the expression of 18S rRNA and CAAT-associated mRNA were analyzed by a split-plot design ANOVA for the randomized complete block design using the GLM procedures (SAS Inst. Inc., Cary, NC). The SH infusion treatment effects were tested in the main plot with individual steers as experimental units, and the tissue distribution effects were tested in the sub-plot with intestinal epithelial regions as experimental units. Sums of squares were partitioned to period, block nested in period, treatment, intestinal each epithelial region, treatment $\times$ epithelial region interaction, and residual error. Random effects were specified for 2 model terms, period and block nested in period. The infusion treatment $\times$ epithelial region interaction was used as the error term for the main plot and the residual error was used as the error term for the sub-plot. Although no treatment $\times$ epithelial region interactions were observed ($P > 0.18$), to avoid masking of potential SH infusion treatment effects on epithelial region, the least squares means associated with infusion treatment were separated by Fisher's LSD ($P \leq 0.05$) within each epithelial region. The probability levels of $P \leq 0.05$ and $0.05 < P \leq 0.10$ were defined as significant differences and tendencies toward differences, respectively.

RESULTS

Development of mRNA Quantification Methodology

All the real-time RT-PCR assays conducted in this study were validated by sequencing their final products. Sequencing results (Figure 1) revealed that the products for $ATB^{0,+}$, $b^{0,+}AT$, $rBAT$, y^+LAT1 , y^+LAT2 , and 4F2hc have 100% identity with their corresponding expected sequences acquired from the virtual template sequences reported in TIGR and Ensembl genomic databases. The sequences of these real-time PCR products now reside in GenBank with accession numbers of EU118972, EU118969, EU118970, EU118966, EU118965, and EU118971, respectively. The sequences of the real-time PCR products of CAT1 mRNA and 18S rRNA were validated previously and reside in GenBank with accession numbers of DQ399522 and EF469831, respectively (Liao et al., 2008a).

Numerous studies have revealed that 18S rRNA expression is very stable and its content can be used as an endogenous control to normalize the expression of

other genes in response to various stimuli (Bustin et al., 2005; Liao et al., 2008a). In this study, we also chose to normalize the relative expression of CAT1, ATB^{0,+}, b^{0,+}AT, rBAT, y⁺LAT1, y⁺LAT2, and 4F2hc mRNA to 18S rRNA expression. Before normalization, however, the use of 18S as a constitutive expression control was validated by evaluating the potential treatment effects on the expression levels of 18S rRNA among 3 small intestinal epithelia. No intestinal epithelial region distribution effect ($0.52 \leq P \leq 0.55$), SH infusion treatment effect ($0.30 \leq P \leq 0.87$), or an interaction between SH infusion treatments and intestinal epithelial regions ($P = 0.65$) on 18S rRNA expression was observed (data not shown). This result is consistent with that found in our previous nucleoside transporter research (Liao et al., 2008b). Accordingly, the 18S rRNA expression levels were used to normalize the relative quantities of the target CAAT-associated mRNA expression by small intestinal epithelia.

Basal Expression of CAAT-Associated mRNA Differed Among Small Intestinal Epithelia

To determine which species of CAAT mRNA are expressed by the small intestinal epithelia of beef steers and to characterize their normal tissue distribution patterns along small intestine epithelial regions, the validated real-time RT-PCR assays were used to determine the presence and relative abundance of 7 mRNA species (CAT1, ATB^{0,+}, b^{0,+}AT, rBAT, y⁺LAT1, y⁺LAT2, and 4F2hc) among duodenal, jejunal, and ileal epithelia collected from the 6 control animals (without SH infusion). Two essential understandings were revealed (Table 2): all CAAT-associated mRNA were expressed by duodenal, jejunal, and ileal epithelia and, for all but y⁺LAT2 and 4F2hc, the relative content of mRNA differed among the epithelia.

Specifically, CAT1 and ATB^{0,+} mRNA shared similar tissue distribution patterns, with duodenal expression

ATB^{0,+}	
TIGR TC297793	831 <u>AGTCGGAGCAGCATTATTTAAAGGAATAAA</u> <u>AGTCTTCTGGAAAGGTGGT</u> GTATTTTACAGCTCTTTTCCCC 900
Product	1 AGTCGGAGCAGCATTATTTAAAGGAATAAAAGTCTTCTGGAAAGGTGGTGTATTTTACAGCTCTTTTCCCC 70
TIGR TC297793	901 <u>TATGTTGCTCTACTCATCCTGGT</u> 923
Product	71 TATGTTGCTCTACTCATCCTGGT 93
b^{0,+}AT	
TIGR TC265966	179 <u>AGAGCAGCGAACCCAAGACCACGAGTCTCCAA</u> <u>AGGAGCTGGGCCTGTT</u> CAGTGGCACCTGCATCAT 245
Product	1 AGAGCAGCGAACCCAAGACCACGAGTCTCCAAAGGAGCTGGGCCTGTTTTCAGTGGCACCTGCATCAT 67
rBAT	
TIGR TC299989	755 <u>CATGACTGTAACCGTGAAATGGTACAACCATAACCA</u> <u>CCCAACAACCTGGTTAAGT</u> GTATATGGAACTCCAGTTGGCACTTTG 836
Product	1 CATGACTGTAACCGTGAAATGGTACAACCATAACCAACCAACAACCTGGTTAAGTGTATATGGAACTCCAGTTGGCACTTTG 82
y⁺LAT1	
TIGR TC264913	707 <u>CATCGCAGGCATTGTTAGACTTGG</u> <u>CCAGGGAGCCTCGACT</u> CACTTTGAAAATTCCTTTGAGGGTTTCATCATTTTCAGTGG 786
Product	1 CATCGCAGGCATTGTTAGACTTGGCCAGGGAGCCTCGACTCACTTTGAAAATTCCTTTGAGGGTTTCATCATTTTCAGTGG 80
y⁺LAT2	
Ensembl ENSBTAT00000016074	498 <u>CCGTCTCCTCGCTGCAG</u> <u>CCTGCATGTGTCTGCTGACATTTGTGA</u> ACTGCGCCTATG 553
Product	1 CCGTCTCCTCGCTGCAGCCTGCATGTGTCTGCTGACATTTGTGAAGTGGCCTATG 56
4F2hc	
Ensembl ENSBTAT00000030071	1206 <u>CCCAACACCTCAGCATCTATAAACATCAGCAT</u> <u>GACTGTGAAGGGCCAGAGT</u> GAGGACCGTAA 1267
Product	1 CCCAACACCTCAGCATCTATAAACATCAGCATGACTGTGAAGGGCCAGAGTGAAGGACCGTAA 62
Ensembl ENSBTAT00000030071	1268 <u>CTCCCTCCTCTCCCT</u> 1282
Product	63 CTCCCTCCTCTCCCT 77

Figure 1. Comparison of the real-time reverse transcription-PCR product sequences (Product) to their respective template sequences retrieved from TIGR and Ensembl databases. Identical base pairs between the corresponding sequences are indicated by “:”; underlines indicate the positions of forward and reverse primers, and the highlights mark the probe positions. These product sequences now reside in GenBank with accession numbers EU118972 for ATB^{0,+}, EU118969 for b^{0,+}AT, EU118970 for rBAT, EU118966 for y⁺LAT1, EU118965 for y⁺LAT2, and EU118971 for 4F2hc.

Table 2. Tissue distribution of cationic amino acid transporter associated mRNA among 3 small intestinal epithelia¹

mRNA	Small intestinal epithelium			SEM ²	P-value ³
	Duodenal	Jejunal	Ileal		
CAT1	2.24 ^a	1.11 ^b	1.02 ^b	0.37	0.065
ATB ^{0,+}	1.45 ^a	0.85 ^b	0.65 ^b	0.14	0.006
b ^{0,+} AT	0.007 ^a	0.043 ^a	1.664 ^b	0.37	0.011
rBAT	1.33 ^a	1.11 ^a	3.96 ^b	0.93	0.089
y ⁺ LAT1	0.68 ^a	0.95 ^{a,b}	1.73 ^b	0.33	0.099
y ⁺ LAT2	1.39	1.04	1.67	0.32	0.391
4F2hc	0.97	0.87	1.17	0.24	0.683

^{a,b}Means within a row that lack a common superscript letter differ (a, b; $P \leq 0.05$).

¹The expression levels of cationic amino acid transporter associated mRNA of 3 regions of small intestinal epithelia were obtained from the control animals (i.e., no starch hydrolysate infusion). The data presented are the ratios of the relative quantities of the cationic amino acid transporter mRNA:18S rRNA.

²SEM = standard error of the mean (n = 6).

³P-values were obtained from ANOVA F-test.

being greater ($P \leq 0.05$) than jejunal or ileal expression but no difference ($P \geq 0.33$) was found between jejunal and ileal expression (Table 2). For CAT1 mRNA, the duodenal expression was approximately 1.0- ($P = 0.05$) and 1.2-fold ($P = 0.04$) greater than the jejunal and ileal expression, respectively, whereas no difference ($P = 0.87$) between the jejunal and the ileal expression was found. For ATB^{0,+} mRNA, duodenal expression was approximately 0.7- ($P = 0.01$) and 1.2-fold ($P = 0.002$) greater than the jejunal and ileal expression, respectively, whereas no difference ($P = 0.33$) was found between jejunal and ileal expression.

The b^{0,+}AT and rBAT proteins function together as a heterodimer to yield system b^{0,+} activity (Palacín et al., 1998). The b^{0,+}AT and rBAT mRNA shared similar patterns of tissue distribution. Specifically, ileal expression was greater ($P \leq 0.07$) than duodenal or jejunal expression, whereas no difference ($P \geq 0.87$) existed between duodenal and jejunal expression (Table 2). For b^{0,+}AT mRNA, ileal expression was approximately 225- ($P = 0.007$) and 38-fold ($P = 0.008$) greater than duodenal and jejunal expression, respectively, whereas no difference ($P = 0.95$) between duodenal and jejunal expression was found. For rBAT mRNA, ileal expression was approximately 2.0- ($P = 0.07$) and 2.6-fold ($P = 0.05$) greater than the duodenal and jejunal expression, respectively, whereas no difference ($P = 0.87$) was found between duodenal and jejunal expression.

Both y⁺LAT1 and y⁺LAT2 bind, individually, to 4F2hc to form heterodimeric functional units that yield specific y⁺LAT1 and y⁺LAT2 transport activities (Dave et al., 2004). The mRNA of y⁺LAT2 and 4F2hc shared similar tissue distribution patterns with no differences ($P \geq 0.39$) existing among duodenal, jejunal, and ileal expression (Table 2). In contrast, ileal expression of

y⁺LAT1 was approximately 1.5-fold greater ($P = 0.04$) than duodenal expression, but not greater ($P = 0.12$) than jejunal expression. Also no difference ($P = 0.56$) was found between duodenal and jejunal expression of y⁺LAT1 mRNA.

In summary, the basal tissue distribution pattern for mRNA encoding CAT1 (monomeric, Na⁺-independent CAA uptake) and ATB^{0,+} (monomeric, Na⁺-dependent CAA, and neutral AA uptake) was duodenal expression > jejunal expression = ileal expression. The basal tissue distribution pattern for b^{0,+}AT and rBAT mRNA was duodenal expression = jejunal expression < ileal expression. The basal tissue distribution pattern for y⁺LAT2 and 4F2hc mRNA was duodenal expression = jejunal expression = ileal expression. The basal tissue distribution pattern for y⁺LAT1 mRNA was duodenal expression = jejunal expression ≤ ileal expression.

SH Infusion Differentially Decreased Some But Not All CAAT mRNA

Neither ruminal nor abomasal SH infusion altered CAT1 mRNA expression by duodenal ($P = 0.33$) or ileal ($P = 0.78$) epithelium of small intestine (Table 3). However, ruminal SH infusion decreased ($P = 0.03$) jejunal CAT1 mRNA expression by 27%, whereas abomasal SH infusion did not affect ($P = 0.13$) jejunal expression of CAT1 mRNA. Neither ruminal nor abomasal SH infusion altered ATB^{0,+} mRNA expression by duodenal ($P = 0.44$), jejunal ($P = 0.35$), or ileal ($P = 0.57$) epithelium of small intestine (Table 3).

Similar to ATB^{0,+} mRNA expression, neither ruminal nor abomasal SH infusion altered b^{0,+}AT mRNA expression by duodenal ($P = 0.30$), jejunal ($P = 0.65$), or ileal ($P = 0.22$) epithelium (Table 3). Also, no ruminal

Table 3. Starch hydrolysate infusion effect on cationic amino acid transporter associated mRNA expression by duodenal, jejunal, and ileal epithelia¹

Item	Starch hydrolysate infusion treatment			SEM ²	<i>P</i> -value ³
	Control	Ruminal	Abomasal		
CAT1					
Duodenum	1.00	0.56	0.76	0.19	0.329
Jejunum	1.00 ^a	0.73 ^b	0.84 ^{a,b}	0.07	0.069
Ileum	1.00	1.00	1.20	0.24	0.781
ATB ^{0,+}					
Duodenum	1.00	1.04	1.32	0.19	0.440
Jejunum	1.00	0.81	0.94	0.09	0.349
Ileum	1.00	1.41	1.37	0.30	0.574
b ^{0,+} AT					
Duodenum	1.00	0.54	0.79	0.19	0.304
Jejunum	1.00	1.28	0.81	0.34	0.646
Ileum	1.00	0.56	1.39	0.30	0.216
rBAT					
Duodenum	1.00	0.84	0.83	0.20	0.770
Jejunum	1.00 ^a	0.59 ^b	0.84 ^{a,b}	0.14	0.197
Ileum	1.00	0.51	1.46	0.38	0.274
y ⁺ LAT1					
Duodenum	1.00	0.71	0.84	0.17	0.542
Jejunum	1.00	0.74	0.70	0.15	0.310
Ileum	1.00	0.67	1.18	0.21	0.286
y ⁺ LAT2					
Duodenum	1.00	0.72	0.79	0.14	0.372
Jejunum	1.00 ^a	0.65 ^b	0.68 ^b	0.09	0.028
Ileum	1.00	0.94	0.85	0.20	0.843
4F2hc					
Duodenum	1.00	0.54	0.81	0.23	0.412
Jejunum	1.00 ^{a,x}	0.61 ^b	0.75 ^{b,y}	0.11	0.070
Ileum	1.00 ^a	0.77 ^{a,x}	1.37 ^{a,y}	0.21	0.169

^{a,b,x,y}Means within a row that lack a common superscript letter differ (^{a,b} $P \leq 0.05$) or tend to differ (^{x,y} $P \leq 0.10$).

¹The relative expression levels of amino acid transporter associated mRNA (CAT1, ATB^{0,+}, b^{0,+}AT, rBAT, y⁺LAT1, y⁺LAT2, and 4F2hc) for the ruminal and abomasal starch hydrolysate infusion treatment groups as well as the control group were all calibrated to the mean of the control group.

²The presented SEM values were pooled (averaged) from that of control ($n = 6$), ruminal ($n = 4$), and abomasal ($n = 6$) infusion treatment groups.

³*P*-values were obtained from ANOVA *F*-test.

or abomasal SH infusion effect on rBAT mRNA expression by either duodenal ($P = 0.77$) or ileal ($P = 0.27$) epithelium was observed (Table 3). However, ruminal SH infusion tended to decrease jejunal rBAT mRNA expression by 41% ($P = 0.08$), whereas abomasal SH infusion did not affect ($P = 0.42$) jejunal rBAT mRNA expression.

Similar to ATB^{0,+} and b^{0,+}AT mRNA expression, neither ruminal nor abomasal SH infusion affected y⁺LAT1 mRNA expression by duodenal ($P = 0.54$), jejunal ($P = 0.31$), or ileal ($P = 0.29$) epithelium (Table 3). In contrast, both ruminal and abomasal SH infusion decreased jejunal y⁺LAT2 mRNA expression by 35% ($P = 0.02$) and 32% ($P = 0.02$), respectively (Table 3). However, there was no ruminal or abomasal SH infusion effect on y⁺LAT2 mRNA expression by either duodenal ($P = 0.37$) or ileal ($P = 0.84$) epithelium.

Ruminal SH infusion decreased jejunal 4F2hc mRNA expression by 39% ($P = 0.03$), whereas abomasal SH infusion tended to decrease ($P = 0.10$) jejunal 4F2hc mRNA expression by 25% (Table 3). Neither ruminal nor abomasal SH infusion affected duodenal 4F2hc mRNA expression ($P = 0.41$). Although the ileal content of mRNA tended to be greater ($P = 0.07$) after abomasal SH infusion than after ruminal SH infusion, neither ruminal ($P = 0.46$) nor abomasal ($P = 0.20$) SH infusion altered ileal 4F2hc mRNA expression.

In summary, ruminal SH infusion decreased ($P \leq 0.08$) jejunal CAT1, rBAT, y⁺LAT2, and 4F2hc mRNA expression by 27 to 41% (Tables 3 and 4), whereas abomasal SH infusion decreased ($P = 0.02$) jejunal y⁺LAT2 expression by 32% and tended to decrease ($P = 0.10$) jejunal 4F2hc mRNA expression by 25%. There was no ruminal or abomasal SH infusion effect ($P >$

0.20) on the duodenal expression of the 7 mRNA species tested.

DISCUSSION

System Activities of CAA Transport in Small Intestinal Epithelia

It is now generally accepted that the capacity for a given free AA (as opposed to peptide-bound AA) to be absorbed across the apical brush border membrane or the basolateral membrane depends on the complement of transport proteins and activities expressed. The transport proteins CAT1, ATB^{0,+}, and b^{0,+}AT have been matched to y⁺, B^{0,+}, and b^{0,+} system activities, respectively, and both y⁺LAT1 and y⁺LAT2 proteins have been matched to y⁺L system activity. For b^{0,+}AT protein to function, a glycoprotein (rBAT) has to be linked (by a disulfide bond) to form a heterodimeric unit. Analogously, y⁺LAT1 and y⁺LAT2 each function as a heterodimer unit with 4F2hc. Studies from laboratory animals have found that the small intestinal absorption of most free AA is achieved by the function of multiple AA transport systems/activities located on brush border and basolateral membranes of epithelial cells (Bergen, 1978; Krehbiel and Matthews, 2003). The potential expression of CAAT transporters by a hypothetical absorptive epithelial cell is depicted in Figure 2.

Transport studies (Guerino and Baumrucker, 1987; Moe et al., 1987; Wilson and Webb, 1990) have revealed that the apical membrane of small intestinal epithelia of cattle possess Na⁺-dependent and Na⁺-independent lysine uptake activities. However, molecular evidence to suggest which CAAT proteins are responsible for these activities has been limited to a single report describing the expression of CAT1 mRNA by duodenal, jejunal,

and ileal epithelia of suckling, weanling, growing, and finishing beef steers (Liao et al., 2008a).

Basal Expression of CAAT mRNA Among Bovine Small Intestinal Epithelia

A primary objective of the present study was to determine the relative tissue distribution pattern of the 7 known mammalian CAAT-associated mRNA among small intestinal epithelia of growing, forage-fed beef steers. Our previous finding that all 3 small intestinal epithelia of growing beef steers express CAT1 mRNA (Liao et al., 2008a) was confirmed by this study. Uniquely, expression of the other 6 genes that encode CAAT by the small intestinal epithelia of growing beef steers was also demonstrated.

The relative absorption capacity for free AA by small intestinal epithelia of laboratory animals has been studied, resulting in the understanding that the jejunum has the greatest capacity for AA absorption (Webb and Matthews, 1994). However, as for other AA, this may not be the case for CAA absorption in the ruminant intestine (Johns and Bergen, 1973; Phillips et al., 1976, 1979). Specifically, the absorption of lysine by sheep intestine has been reported to increase with distance from the pylorus and be maximal in the ileum (Johns and Bergen, 1973). Also, the total ileal lysine uptake capacity (per mg of brush border membrane vesicle) in Holstein steers was found to be greater than jejunal uptake (Wilson and Webb, 1990).

The differential expression profiles for CAAT mRNA found in the present study may support this understanding. That is, although the present study (Table 2) found that ileal mRNA expression was greatest for only b^{0,+}AT, rBAT, and y⁺LAT1, apical system b^{0,+} activity (mediated by the b^{0,+}AT/rBAT heterodimer) plays a major role in small intestinal absorption of CAA from

Table 4. Basal and regulated expression of cationic AA transporter (CAAT) associated mRNA by small intestinal epithelia¹

CAAT activity	mRNA	Basal expression ²	Starch infusion effect ³
y ⁺	CAT1	D > J = I	J decreased by R**
B ^{0,+}	ATB ^{0,+}	D > J = I	No R or A effect
b ^{0,+}	b ^{0,+} AT	D = J < I	No R or A effect
	rBAT	D = J < I	J decreased by R*
y ⁺ L	y ⁺ LAT1	D = J, J = I, D < I	No R or A effect
	y ⁺ LAT2	D = J = I	J decreased by R** or A**
	4F2hc	D = J = I	J decreased by R** or A*

¹mRNA expression by duodenal (D), jejunal (J), and ileal (I) epithelia.
²Data obtained from the control animals. “ = ” indicates no difference (P > 0.10) between small intestinal epithelial regions; “ > ” indicates a greater mRNA expression (P ≤ 0.05), whereas “ < ” indicates a lower mRNA expression (P ≤ 0.05).
³The effect of ruminal (R) or abomasal (A) starch hydrolysate infusion (vs. water infusion) on D, J, or I epithelial mRNA expression; **P ≤ 0.05; *P ≤ 0.10.

Figure 2. A hypothetical mammalian model for the 7 known mammalian cationic AA transport-associated proteins expressed by small intestinal absorptive epithelial cells. The subcellular location and predominant direction of substrate flow (arrows) are depicted for each functional transport unit and were derived from data reviewed by Devés and Boyd (1998), Krehbiel and Matthews (2003), Dave et al. (2004), and Bröer (2008). AA⁺ = cationic AA; AA^{0,+} = cationic and neutral AA; CysC = cystine.

digesta (Bröer, 2008). Furthermore, y⁺LAT1 basolateral activity facilitates transepithelial passage of apically absorbed CAA into the blood (Figure 2). Moreover, it is of physiological importance to realize that the small intestine epithelial region that has the greatest reported total and CAA absorptive capacity (ileum) also expresses the greatest amount of mRNA that encode CAA transporters (b^{0,+}AT, rBAT, y⁺LAT1) that transport CAA in exchange for neutral AA (Figure 2). Also, although the expression of CAT1 and ATB^{0,+} mRNA was greatest in the duodenum, the physiological significance of this observation is unknown given the relatively small amount of duodenal vs. jejunal and ileal epithelial mass.

Although the quantitative correlations among CAAT activities, proteins, and their associated mRNA have not been thoroughly studied, cell culture models of CAT1 function have shown that mRNA expression is proportional to CAT1 activity (Hatzoglou et al., 2004). Also, in mice, the relative expression of b^{0,+}AT, y⁺LAT1, and 4F2hc protein parallels mRNA expression (Dave et al., 2004). Ultimately, however, the physiological consequence of the relative distribution of CAAT mRNA by small intestinal epithelia of cattle awaits

validation by CAAT protein expression and function experimentation.

Regulated Expression of CAAT mRNA in Bovine Small Intestinal Epithelia

The second objective of this study was to evaluate the effects of ruminal and abomasal SH infusion on the expression of CAAT-associated mRNA by small intestinal epithelia. Ruminal supplementation of high-quality forage diets with energy concentrates (such as corn starch) can increase the production of ruminal microbes, and hence the delivery of microbial AA to the small intestinal lumen (Taniguchi et al., 1995; Walker and Harmon, 1995; Huntington, 1997; Bach et al., 1999; Elizalde et al., 1999). An increased delivery of AA will lead to a proportionally increased luminal supply of CAA, the substrates of CAAT. Because the absorption of CAA by CAAT-mediated transport events are energy demanding (ultimately depending on Na⁺/K⁺ ATPase function) and the expression of CAAT is thus limited by the energy status of cells, we included infusion of SH into the abomasum to provide an increased luminal energy supply to the small intestine.

Results from studies designed to establish the effects of substrate supply on the capacity for intestinal CAA absorption are equivocal (Karasov et al., 1987; Palacio and Torres y Torres, 1992; Devés and Boyd, 1998; Christie et al., 2001). In the present study, adding SH to the rumen (hence, increasing microbial protein/CAA supply) reduced the contents of CAT1, y^+LAT2 , and 4F2hc mRNA. Thus, the capacity for y^+ and y^+L system-mediated absorption of CAA may have been reduced. These results are in keeping with the adaptive repression theory (Ferraris and Diamond, 1989; Hatzoglou et al., 2004). That is, the down-regulation of CAT1, y^+LAT2 , and 4F2hc expression would reduce the potential for absorbing the unnecessary amounts of CAA (lysine, arginine, and histidine), thereby avoiding the energy cost (and perhaps the toxicity) of metabolizing excess CAA. Why $ATB^{0,+}$, $b^{0,+}AT$, and y^+LAT1 mRNA expression was not down-regulated by ruminal SH infusion is not clear. However, it is known that both $B^{0,+}$ and $b^{0,+}$ systems have broader substrate specificity with high affinity not only for CAA but also for small and large neutral AA. Some investigators believe that systems $B^{0,+}$ (Bröer, 2008) and $b^{0,+}$ (Devés and Boyd, 1998) do not play major roles in small intestinal CAA absorption in animals other than rabbit. Although both y^+LAT1 and y^+LAT2 proteins possess y^+L system activity, y^+LAT1 has a narrower tissue distribution but a broader substrate specificity (Bröer et al., 2000). This difference may explain why only y^+LAT2 , and not y^+LAT1 , mRNA expression was affected by ruminal SH infusion treatment.

As to abomasal SH infusion, our results suggest that the y^+L system activity performed by the $y^+LAT2/4F2hc$ heterodimer in the jejunal region might be reduced by the presence of high levels of SH or glucose. As such, these results are consistent with the report that Na^+ -dependent uptake of CAA by small intestinal brush border membrane vesicles of rats can be inhibited by the presence of glucose (Wolfram et al., 1984).

CONCLUSIONS

This study found that the mRNA encoding 7 mammalian CAAT-associated proteins (CAT1, $ATB^{0,+}$, $b^{0,+}AT$, rBAT, y^+LAT1 , y^+LAT2 , 4F2hc) are expressed throughout the small intestinal epithelia of growing beef steers, indicating that ruminating cattle possess at least 4 system activities responsible for CAA absorption via small intestinal epithelia. This understanding appears to be consistent with the limited reports identifying cattle-specific Na^+ -dependent and independent lysine uptake activities. Although limiting CAA supply to forage-fed cattle is thought to limit growth, increasing the luminal supply of microbe-derived AA (by rumi-

nal supplementation of SH) either decreased (CAT1, rBAT, y^+LAT2 , 4F2hc) or did not affect transcription products of genes encoding CAAT proteins. Similarly, increasing the supply of glucose (by abomasal supplementation of SH) to the small intestinal lumen either decreased (y^+LAT2 , 4F2hc) or did not affect CAAT mRNA content of epithelia. Thus, assuming that CAAT mRNA content represents CAAT functional capacity, this study has demonstrated that increasing the small intestinal luminal supply of CAAT substrates results in reduced capacity for both apical and basolateral membrane transport of CAA, whereas increasing luminal glucose supply (thus, increasing the potential energy status of small intestinal epithelia that express CAAT) reduces only basolateral CAA transport capacity.

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