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UT-B is expressed in bovine rumen: Potential role in ruminal urea transport

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ABSTRACT

The UT-A (SLC14a2) and UT-B (SLC14a1) genes encode a family of specialized urea transporter proteins that regulate urea movement across plasma membranes. In this report we describe the structure of the bovine UT-B gene and characterize UT-B expression in bovine rumen. Northern analysis using a full-length bovine UT-B probe detected a 3.7 kb UT-B signal in rumen. RT-PCR of bovine mRNA revealed the presence of two UT-B splice variants, bUT-B1 and bUT-B2, with bUT-B2 the predominant variant in rumen. Immunoblotting studies of bovine rumen tissue, using an antibody targeted to the N-terminus of mouse UT-B, confirmed the presence of 43-54 kDa UT-B proteins. Immunolocalisation studies showed that UT-B was mainly located on cell plasma membranes in epithelial layers of the bovine rumen. Ussing chamber measurements of ruminal trans-epithelial transport of $^{14}$C-labelled urea indicated that urea flux was characteristically inhibited by phloretin. We conclude that bUT-B is expressed in the bovine rumen and may function to transport urea into the rumen as part of the ruminant urea nitrogen salvaging process.
INTRODUCTION

In recent years, the theory that urea simply diffuses across cell membranes has been dispelled by the discovery of facilitative urea transporters in many tissues displaying high urea permeabilities, such as the renal inner medullary collecting duct (reviewed in references 23, 19). Facilitative urea transporters are derived from the UT-A (Slc14a2) and UT-B (Slc14a1) genes, and have been shown to play a vital role in the urinary concentration mechanism (6, 1). Six UT-A gene splice variants have been characterized: UT-A1 (20), UT-A2 (22), UT-A3 and UT-A4 (13), UT-A5 (4), and UT-A6 (24). In contrast, UT-B gives rise to only two transcripts that are thought to encode the same protein (16). When expressed in Xenopus oocytes, all UT-A and UT-B proteins transport urea in a phloretin-inhibitable manner (23).

Although initially cloned from kidney (29), facilitative urea transporters have also been identified in several tissues, including brain (2), testis (5) and the gastrointestinal tract (11, 26). Urea transporters expressed in the gastrointestinal tract have been suggested to mediate urea flux into the intestinal lumen as part of the process of urea nitrogen salvaging (UNS) (26). Ruminant animals, such as cattle and sheep, subsist on a diet that is high in cellulose and low in protein nitrogen. As a result, there is a need to recycle nitrogen and hence UNS is vital for maintaining nitrogen balance in ruminants. During UNS, 40-80% of the urea produced in the liver passes into the digestive tract and is broken down by resident bacteria into ammonia and carbon dioxide (15). The bacteria use ammonia to synthesize the amino acids and peptides required for growth, which along with the ammonia can be reabsorbed
by the ruminant host, thus completing the ‘salvaging’ of urea nitrogen (7). The molecular basis of ruminal urea entry has to date not been resolved. Since ruminants account for a significant portion of biomass on earth, understanding how they process nitrogen has widespread implications.

In this study, we determined the structure of the bovine UT-B gene, identified and characterized bovine UT-B cDNAs, and showed that bUT-B protein is expressed in rumen epithelia. We conclude that UT-B is present in the bovine rumen and may participate in urea transport across rumen epithelia as part of the urea nitrogen salvaging process.
METHODS

**Bovine UT-B gene structure** - The Trace Archive of the bovine genome project at the National Center for Biological Information (NCBI), containing raw reads from the first 3-fold genome coverage (approximately 12 million reads at the time of screening), was searched via BLASTN using the full insert sequence of bovine UT-B cDNA (GenBank Acc: AY624602). Trace files whose sequence showed highly significant matches to the cDNA (scores >300), as well as the mate-pair end sequences from the respective clones, were collected in a directory and used to construct initial genomic contigs via phred (3) and phrap (9) algorithms. Contig sequences were masked for repetitive elements using RepeatMasker (21), and used to search for overlapping trace files in the archive, which were added to the directory for reconstruction of contigs. The process was repeated until none of the contigs in the phrap output identified trace files not already in the directory. This resulted in construction of three contigs containing portions with exact matches to the exons of the bovine UT-B1 cDNA, leaving two gaps in the gene sequence. Primers were designed to span the gaps by PCR, and sequence was obtained by amplification of a bovine BAC (clone 48L6) from the CHORI-240 library (BACPAC Resources Inc., Oakland CA) and sequencing of the products with the amplification primers, nested primers, or both. The resulting 27,657 bp contig was edited by manual inspection using the consed viewing program (9), and areas of low sequence quality or areas where read overlap was exclusively from low complexity sequence were targeted for finishing using additional PCR-based amplification and sequencing. The final genome sequence has been submitted to GenBank with accession number
AY838799 and encompasses the entire cDNA sequence with appropriate splice boundaries and polyA addition signal.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR)** – Poly (A⁺) RNA was obtained from bovine rumen and kidney using an oligo(dT)-cellulose batch method and 1µg used to produce cDNA via reverse transcription (Superscript II, Invitrogen, USA). PCR amplification with a *Taq* polymerase enzyme (Roche, USA) was performed on cDNA from clone AY624602 (positive control), bovine rumen and bovine kidney using bUT-B specific primers. The forward primers were (a) Primer 1 (5’-TGCCTAACATAACGAGTTC-3’) designed against AY624602 37-55bp and (b) Primer 3 (5’-AGGGCTACAACGCTACCCTGGTG-3’) designed against AY624602 424-444bp; the reverse primers were (a) Primer 2 (5’-ATAGTACAGTCTTAGTGG-3’) designed against AY624602 1470-1489bp, and (b) Primer 4 (5’-GAAGATGCCCTGCTCCACGG-3’) designed against AY624602 774-794bp. Cycling parameters were: initial denaturation 94°C for 2 min., followed by 30 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, The final extension was at 72°C for 8 min. The products obtained from PCR of bovine rumen cDNA were investigated through direct sequencing (Lark Technologies, UK).

**Southern Analysis** – After electrophoresis of PCR products through a 1% agarose gel, the gel was denatured with 0.5 M sodium hydroxide and then neutralised with 1.5 M NaCl plus 0.5 M Tris HCl. The PCR products were capillary-transferred to Hybond-N filters and then probed with ³²P-labelled bovine UT-B1 at high stringency (final wash at 65°C in 0.1X SSC, 0.1% SDS).
Northern Analysis - To investigate the distribution of bovine UT-B urea transporter transcripts, poly (A+) RNA was isolated from bovine kidney, bovine rumen and mouse kidney. Poly (A+) RNA was obtained using an oligo(dT)-cellulose batch method (4). Poly (A+) RNA (3 µg/lane) was separated in a 1% agarose gel in the presence of 2.2 M formaldehyde and transferred to Hybond-N filters (Amersham Pharmacia Biotech). Filters were probed with a radioactive $^{32}$P-labelled probe consisting of full-length bUT-B1 cDNA (GenBank Acc: AY624602) or mUT-A1 cDNA (GenBank Acc: AF366052). Hybridization was for 16 hours at 42°C (50% formamide) and washing at 65°C (high stringency) in 0.1X standard sodium citrate (SSC), 0.1% sodium dodecyl sulphate (SDS). Autoradiographs were produced using Biomax MS Film (Kodak, USA).

Xenopus oocyte expression experiments – These experiments were performed as previously described (22). A plasmid containing the bovine UT-B1 clone (GenBank Acc: AY624602), obtained from Dr. T. Smith (USDA/ARS U.S. Meat Animal Research Center, P.O. Box 166, Clay Center, NE, USA), was linearized using Not I. Complementary RNA was prepared using the Sp6 mMessage mMachine RNA kit (Ambion, USA). For bUT-B2, the 1600bp ruminal UT-B PCR product was sub-cloned into the TOPO2.1 vector (Invitrogen, USA), cDNA obtained using the Qiagen Miniprep kit (Qiagen), and then linearised using Hind III. Complementary RNA was prepared using the T7 mMessage mMachine RNA kit (Ambion, USA). Oocytes were injected with water, bovine UT-B1 cRNA (~1 ng per oocyte), bovine UT-B2 cRNA
(~1ng per oocyte) or mouse UT-B1 cRNA (~1ng per oocyte), and then incubated at 18°C for 3 days. [¹⁴C] urea uptake was then measured as previously described (22).

**Antisera** - To study the distribution of UT-B proteins in bovine rumen, we utilised the characterized polyclonal MUTB antibody, previously used to detect the mouse UT-B facilitative urea transporter (26). The antiserum had been affinity purified using Affigel support columns (Biorad) containing immobilized immunizing peptide. Antiserum MUTB was raised in rabbits to amino acids 1 to 19 (H₂N-MEDSPTMVKVDRGENQILS-CONH₂) of mouse UT-B (GenBank Acc: AJ420967). Characterized antisera (ML446, MQ2 and ML194), previously used to detect mouse UT-A facilitative urea transporters (26), were also used.

**Immunoblotting** - Rumen samples were obtained from commercial slaughterhouses and were excised from the forestomachs of cattle within 15-40 minutes of slaughter. Approximately 100cm² of rumen from the ventral sac, in the region 10 cm from the left longitudinal groove, were immediately washed and then transported in ice cold organ transplantation preservation solution (140 mM sucrose, 42.3 mM Na₂HPO₄, 26.7 mM NaH₂PO₄; pH 7.4). The epithelial mucosae were then stripped from the muscle layers using arterial forceps scissors and scalpel, snap frozen in liquid nitrogen and stored at ~80°C. Male adult NMR1 mice were killed by cervical dislocation and kidneys removed immediately. Rumen and kidney samples were homogenized in ice-cold buffer with a hand held dounce homogenizer. The homogenization buffer (pH 7.6) contained 12 mM HEPES, 300 mM mannitol and
several peptidase inhibitors added immediately before use – 1 µg/ml Pepstatin, 2 µg/ml Leupeptin and 1 µg/ml phenylmethanesulfonyl fluoride (Sigma). Homogenates were initially centrifuged at 2,500g for 15 min. at 4°C. The resulting supernatant was centrifuged at 200,000g for another 30 min. at 4°C. These plasma membrane enriched pellets were retained and resuspended in homogenization buffer. Total protein concentrations were determined using a Biorad Protein Assay Reagent Kit (Biorad). 5X reducing Laemmli sample buffer (5% SDS, 25% glycerol, 0.32 M Tris(hydroxymethyl)aminomethane pH 6.8, bromophenol blue, 5% β-mercaptoethanol) was added to protein samples in a ratio of 1:4, which were then heated at 60°C for 15 min. SDS-PAGE was performed on minigels of 10% polyacrylamide by loading 20 µg/lane of protein. Proteins were then transferred electrophoretically to nitrocellulose membranes (Gelman Sciences). After blocking with 5% non-fat dry milk in washing buffer (15 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.01% Tween-20) for 1 hour, the membranes were probed with affinity-purified MUTB antiserum for 16 hours at 4°C. The membranes were rinsed in washing buffer for 3 x 10 min., then probed with goat anti-rabbit horseradish peroxidase (HRP) linked secondary antiserum (Dako, UK) at 1:5000 dilution in 5% non-fat milk in washing buffer for 1 hour. After another 3 x 10 min. rinse in washing buffer, detection of protein was performed using the ECL Western Blotting Detection Reagents and ECL film (Amersham Pharmacia, UK).

**Immunocytochemistry** - Rumen papillae were isolated directly from stripped rumen epithelial mucosae using scissors. Papillae were immersion fixed in -20°C methanol, acetone or ice-cold 3% paraformaldehyde in PBS for a minimum of 4 hours or
overnight. Papillae were then incubated for 24 hours in 30% sucrose in PBS at 4°C overnight before embedding in OCT (Tissue-Tek, Miles Laboratories, Naperville Illinois) and cryo-sectioning. 5 µm sections were washed three times with PBS, permeabilised with 0.1% saponin in PBS for 20 minutes then blocked with 10% serum (species dependent on host animal used for secondary antibody production) in PBS. Sections were then incubated overnight at 4°C with primary antibody (typically diluted 1:50 in 10% serum/PBS), washed three times then incubated for 1 hour at room temperature with an appropriate Alexa Fluor conjugated secondary antibody (Molecular Probes). Typically, sections were counterstained with ethidium homodimer I (Molecular Probes) to highlight the nuclei. Immunofluorescence signals were detected using confocal laser scanning microscopy and all immunocytochemistry was repeated for material obtained from at least 3 animals.

**Ussing chamber experiments** - Isolated bovine ruminal sheets were mounted in thermostated (37°C) Ussing chambers (1.76cm² exposed area) for measurement of transepithelial [¹⁴C]-urea and [³H]-mannitol fluxes in the absorptive (J_lumen to blood) and secretory (J_blood to lumen) directions. A modified Ringer’s solution was used (all mM): NaCl (80), NaHCO₃ (25), Na acetate (40) CaCl₂ (2.5), MgSO₄ (3), KH₂PO₄ / K₂HPO₄ (2.8), glucose (10), with gassing by 95%O₂/5%CO₂ (pH 7.4, 37 °C). Na acetate is present to partially mimic rumen anion composition and provide an alternative energy source (8). Mannitol and urea were added to give a total unlabelled concentration of 1mM. The composition of the Ringer’s in the apical (lumen) and basal (blood) chambers (7cm³ total volume) was identical. [¹⁴C]-urea (0.1 µCi.ml⁻¹) and [³H]-mannitol (0.2 µCi.ml⁻¹) were added to either the apical or basal chamber, and in each case an
equivalent concentration of unlabelled substrate was present in the contralateral chamber. The tissues were equilibrated for 10 minutes, when 0.2cm$^3$ samples from both apical and basal chambers were taken. Two flux periods of 30 minutes with further sampling were then undertaken. Phloretin (0.1M in DMSO) was added at 40 minutes to either apical or basal bathing solutions to give a final concentration of 1mM. Fluxes were expressed as nmol.cm$^{-2}$.hr$^{-1}$.

Statistics – for statistical analysis of oocyte expression experiments, one-way analysis of variance (ANOVA) was used. If the ANOVA indicated a difference, treatment comparison between groups with the Student-Newman-Keuls post hoc test was performed. Groups were deemed statistically significant if $P<0.05$. Linear regression was performed by the method of least squares (Sigmaplot, SPSS).
RESULTS

Structure of the $bUT-B$ gene

The human $Jk$ gene (encoding UT-B) lies at 41.5 Mb on chromosome 18 (HSA18) genome sequence (NCBI Build 35.1). HSA18 has been shown to have extensive conserved synteny with bovine chromosome 24 (BTA24)(24), and a BAC fingerprint contig predicted to span the gene (based on comparison of BAC end sequences to the human genome) has been mapped to 50-55 centimorgans (cM) on the BTA24 linkage map (12). Using trace files from the bovine genome sequence effort ongoing at the Baylor College of Medicine, we were able to construct a bovine genome sequence spanning the locus with the few gaps in the sequence filled by PCR-based sequencing of a BAC clone containing the gene. The resulting 27 kb contig (GenBank accession number AY838799) revealed that the bovine UT-B ($bUT-B$) gene consisted of 10 exons spanning 20kb (see Figure 1). Exon sizes ranged from 50bp (exon 9) to 2036bp (exon 10 to first poly adenylation site). Exon 1 encoded the 5' untranslated sequence. Translational start codons were identified in exons 2 and exon 3 and an in-frame stop codon was identified in exon 10.

Differential splicing of exons results in two splice forms (see Figure 1) that differ with respect to the site of translational initiation. Consequently, the splice products differ with respect to the N-terminal amino acids (see below). Direct sequencing of introns revealed all exon/intron junctions contain canonical 5'-donor-gt and the 3'-acceptor ag sequences (Table 1). Introns ranged in size from 204bp to 4841bp. Comparison of the structure of the $bUT-B$ gene with the $Jk$ gene (human UT-B) identified a considerable conservation of structure. Except for the 5'UTR of the human gene,
which spans three exons compared to one in the bUT-B gene, the rest of the gene was relatively similar.

**Bovine UT-B cDNAs**

Searching a *Bos taurus* clone library (MARC4BOV, derived from whole embryos, BACPAC Resources, USA) for clones homologous to human UT-B (Genbank Acc: NM015865) we identified a 5' EST (EST id: 153419, GenBank Acc: BE665260). Direct sequencing of this cDNA revealed that it had a high degree of identity to human UT-B. The cDNA was 3133 bp in length (GenBank Acc: AY624602), with a predicted open reading frame (ORF) between nucleotides 75 and 1229. This ORF encodes a 384 amino acid protein, bovine UT-B (bUT-B1), which has 85% identity with mouse UT-B (GenBank Acc: CAD12807) and 79% identity with human UT-B (GenBank Acc: Q13336) (see Figure 2). In comparison, bUT-B1 has only 62% identity with human UT-A2 (GenBank Acc: CAA65657). No UT-A cDNAs were apparent in the MARC4BOV library.

**RT-PCR**

RT-PCR experiments using primer sets 1 & 4 and 1 & 2 both gave products with rumen cDNA that were slightly larger than those obtained with bUT-B1 and kidney cDNA (see Figures 3A and 3B), whereas primers 3 & 4 did not (see Figure 3C). These larger 900 and 1600 bp ruminal products were initially confirmed as bUT-B after they were detected during Southern blotting with a bUT-B1 probe (see Figure 3D). Direct sequencing of these products showed that 156 additional base pairs were present in the 5' region. Importantly, this new sequence was present in the bUT-B
gene, 3’ to exon 1 and 5’ to exon 3. Interestingly, splicing in of these nucleotides (exon 2) was predicted to introduce an in-frame ATG that was 5’ to that present in clone AY624602. The additional nucleotides encoded 55 amino acids and, apart from these, the protein was predicted to be identical to bUT-B1 protein. This longer rumen bUT-B variant, with 439 amino acids in total, we classified as bUT-B2 (see Figure 2). Analysis of both variants using SignalP [http://www.cbs.dtu.dk/services/SignalP-3.0/] showed that the N-termini of bUT-B1 and bUT-B2 were unlikely to contain cleavable signal peptides.

**Southern Analysis**

Southern analysis of RT-PCR ~1600bp and ~900bp ruminal products, generated by primer sets 1 & 2 and 1 & 4 respectively, showed that they corresponded to bUT-B2. In addition, a second, weaker signal ~150bp smaller was present with primer set 1 & 4 (Figure 3D) and corresponds to bUTB1. It therefore appears that although the predominant isoform in the rumen is bUT-B2, bUT-B1 is also present.

**Northern Analysis**

Using a full-length bUT-B1 cDNA probe (AY624602), high stringency northern analysis revealed a 3.5kb mRNA signal in bovine kidney and a slightly larger 3.7kb signal in bovine rumen (see Figure 3E). These results indicate that bUT-B is expressed in both kidney and rumen, though the predominant ruminal transcript appears to be ~0.2kb longer, corresponding to the difference between bUT-B1 and bUT-B2. These results also suggest that the cDNA AY624602 is lacking up to 0.5kb
of untranslated sequence. In contrast to these results, no signals were detected when the UT-A probe AF366052 was used (data not shown).

**Xenopus oocyte expression**

In order to test that bUT-B proteins were functional transporters, we expressed bUT-B1 and bUT-B2 cRNA in *Xenopus* oocytes. Expression of bUT-B1 cRNA induced a 4-fold increase in urea transport compared to water-injected controls (P<0.001, ANOVA, see Figure 4A). This increase in urea transport was significantly inhibited by 500µM phloretin (P<0.01, ANOVA, see Figure 4A). Expression of bUT-B2 cRNA induced a 2-fold increase in urea transport compared to water-injected controls (P<0.01, ANOVA, see Figure 4A), which was again significantly inhibited by 500µM phloretin (P<0.05, ANOVA). Thionicotinamide (2mM), another known inhibitor of facilitative urea transporters (13), also inhibited the urea transport induced by bUT-B1 (P<0.01, ANOVA, see Figure 4B), to a similar degree as phloretin. Indeed, the results obtained for bUT-B1 were very similar to those obtained with mUT-B1 (see Figure 4B). Therefore, both bUT-B1 and bUT-B2 cDNAs encode functional, phloretin-inhibitable urea transporters.

**Western analysis**

Western analysis was performed using MUTB, a previously characterized antibody raised to the amino-terminal end of mouse UT-B (26). In both mouse kidney and bovine rumen, MUTB detected a strong 43-54 UT-B kDa signal (see Figure 5), similar to the 41-54 kDa UT-B signal reported in rat kidney (27). Prior incubation of the MUTB antibody with ~0.05 mg/ml of the initial immunizing peptide abolished the
43-54 kDa signal in mouse kidney and markedly reduced the ruminal signal (see Figure 5). The residual signal indicated that, while the majority of the 43-54 UT-B kDa smear corresponded to bUT-B, a minor component was due to a non-specific, non peptide-blockable signal. Differential centrifugation was used to separate plasma membranes and intracellular vesicles from cytosolic proteins. Using semi-quantitative immuno blotting, the ruminal UT-B signal was found to be stronger in the 200,000g pellet fraction than in the 200,000g supernatant fraction, indicating that the UT-B protein was predominantly located in the plasma membranes and intracellular vesicles rather than the cytoplasm. Indeed, the majority of the supernatant signal was not associated with UT-B. Finally, in contrast to MUTB, using a battery of UT-A targeted antibodies (ML446, MQ2, ML194) detected no signals (data not shown).

**Immunolocalisation**

Immunolocalisation studies clearly showed widespread MUTB staining within the bovine rumen epithelium. MUTB staining appeared to be both plasma membrane and variably intracellular. Staining was present in cells of the stratum granulosum, the stratum spinosum and the stratum basale. In contrast, MUTB did not stain the stratum corneum (see Figures 6A & 6B). MUTB staining was also present in the vascular tissue and weakly present in connective tissue. All MUTB staining was abolished after prior incubation with the immunizing peptide (see Figure 6B - INSET).

**Trans-epithelial Flux Experiments**

Taken altogether, the molecular and immunological data above illustrated the presence of bUT-B in rumen. To determine whether this protein may play a role in
trans-ruminal urea transport we performed functional studies to measure bi-
directional fluxes using rumen mucosae mounted in Ussing chambers. Bi-directional
14C-labelled urea fluxes at a total concentration of 1mM were similar ($J_{\text{lumen to blood}}$
22.5 ± 3.5 nmol.cm$^{-2}$hr$^{-1}$ (SEM), n=7 measurements from 4 animals, $J_{\text{blood to lumen}}$
16.6± 3.6 nmol.cm$^{-2}$hr$^{-1}$ (SEM), n=7 as above) with no evidence of a substantial
active net absorption (p>0.2, for $J_{\text{lumen to blood}}$ vs $J_{\text{blood to lumen}}$). Transepithelial mannitol
fluxes were used to determine the magnitude of non-cellular passive permeation
(paracellular) pathways. In control conditions, 14C-urea fluxes exceeded
simultaneously measured $^3$H-mannitol fluxes (both at a total concentration of 1mM)
by 2.5 fold and 1.97 fold for $J_{\text{lumen to blood}}$ and $J_{\text{blood to lumen}}$ respectively. A scattergraph
of simultaneously measured urea and mannitol fluxes (see Figure 7A) demonstrates
that trans-epithelial 14C-labelled urea flux varies concurrently with $^3$H-mannitol flux,
suggesting that a component of trans-epithelial urea flux is mediated by a non-
cellular, paracellular pathway. Importantly, however, phloretin (1mM) inhibited a
component of the trans-epithelial urea flux (see Figure 7A), and significantly changed
the urea to mannitol flux ratio from 2.8 +/- 0.2 (n=14) in control conditions to 1.8 +/-
0.2 (n=14) (P<0.01, t-test) (see Figure 7B). These findings suggest a role for a
 cellular trans-epithelial pathway for urea mediated by a phloretin-sensitive
transporter. The fact that phloretin fails to completely suppress bUT-B mediated flux
in oocytes (see above) suggests that a definite partition of 14C-labelled urea flux
between a cellular mediated and a paracellular route cannot be made. In any case
the magnitude of the “paracellular” route is variable (see Figure 7A) and may be
enhanced in vitro (see discussion).
DISCUSSION

In this study we set out to determine the structure of the bovine UT-B gene, characterize bovine UT-B and investigate the possible role of bUT-B in ruminal urea transport.

We determined the structure of the UT-B gene and discovered that it was very similar to the human UT-B gene. Comparative genomic analysis indicated that exon 2 had not been described previously and was present in the human UT-B gene. This raised the possibility that UT-B may exist as two isoforms in human. Clearly, this needs further investigation, possibly making use of primer sets homologous to those that we have developed in the present study.

By performing BLASTn database searches we identified a 5' EST with homology to human UT-B. Direct sequencing of this cDNA and functional analysis using Xenopus oocytes confirmed that, characteristic of facilitative urea transporters, the encoded protein transported urea and was phloretin-inhibitable. Although a previous study by Ritzhaupt et al. reported that a cDNA fragment amplified from sheep rumen cDNA was homologous to rat kidney UT-B, they did not report isolation of a full-length clone (18). Therefore, our study is the first to characterize a functional urea transporter from a ruminant animal. In this current study we also discovered a splice variant of bUT-B, namely bUT-B2. bUT-B2 was predicted to be identical to UT-B1, but due to the splicing-in of an in-frame ATG has an additional 55 N-terminal amino acids. This novel 55 amino acid sequence does not appear to contain any additional phosphorylation sites or any targeting motifs, so its precise function is as yet unclear. Importantly, however, expression of bUT-B2 cDNA in Xenopus oocytes
confirmed that this novel isoform encoded a functional, phloretin-sensitive transporter. Previously, the human UT-B gene has been suggested to encode a 4.4kb and a 2.0kb UT-B transcripts that differ in length due to the use of alternative polyadenylation, but that encode the same protein (16). Using Northern analysis we showed the presence of a 3.7 kb mRNA UT-B transcript in the bovine rumen and a 3.5kb UT-B transcript in the bovine kidney. The presence of additional sequence in the ruminal transcript compared to bUT-B cDNA was also confirmed by the RT-PCR results. We suggest that bUT-B1 is likely to be encoded by the 3.5kb transcript and is the predominant renal UT-B isoform. Furthermore, the predominant ruminal UT-B transcript is 3.7kb and is likely to encode UT-B2.

Immunolocalisation studies provided an interesting insight into the possible role of UT-B in bovine rumen. Bovine UT-B was present in the plasma membranes of cells in the stratum basale, spinosum and granulosum, but not in the outermost cell layer, the stratum corneum. Recently a functional model of the rumen epithelium has been suggested on the basis of the expression of junctional markers, gap junctions and the Na⁺-K⁺ ATPase; it is likely that the epithelial permeability barrier (zonae occludentes) occur at the level of the stratum granulosum, the cells of the stratum granulosum, spinosum and basale form a functional syncitium interconnected by gap-junctions, and that the Na⁺-K⁺ ATPase is concentrated in the stratum basale (10). The overall distribution of bUT-B in the plasma membrane of all 3 cell layers suggests that it will mediate flux of urea across the functional thickness of the rumen epithelium (stratum basale, spinosum and granulosum); lack of expression of bUT-B in the stratum corneum correlates with an absence of junctional occluding junctions in this cell layer, indicating that the stratum corneum may not restrict trans-ruminal
urea flux. The stratum corneum is subject to mechanical abrasion by rumen contents, so that its primary role is that of mechanical protection.

Using the Ussing chamber method, functional analysis showed that trans-epithelial urea flux was bi-directional, as would be expected if transcellular urea transport were via facilitated diffusion through UT-B proteins. Importantly, the flux of urea was reduced by phloretin, implicating a UT-A or UT-B transporter in the movement of urea. Indeed, our findings agree with a previous report on sheep rumen epithelia (17), in which urea transport was reduced by phloretin, and by thiourea – another known inhibitor of facilitative urea transporters (28). Our data suggest that ruminal urea transport occurs via UT-B proteins, since bovine UT-A proteins could not be detected by either Northern or western analysis.

Metabolism of urea by resident bacteria plays an important role in maintaining nitrogen balance in ruminants (15). The rumen is a specialized organ anterior to the small intestine. The rumen is home to a plethora of commensal bacteria that facilitate digestion of a diet that is high in fibre. In addition these bacteria metabolise urea, and in so doing make urea nitrogen available for absorption by the host. In ruminants, 40-80% of the urea produced in the liver passes into the digestive tract and is broken down by resident bacterial into ammonia and carbon dioxide (15). Our results suggest that UT-B could play a role in this entry of urea into the ruminant digestive tract.

The action of bacterial urease in the rumen renders ruminal urea to low levels and, combined with the maintained high levels of urea in the blood, implies that the net urea concentration gradient would be expected to be from blood to rumen. Interestingly, since facilitative transporters only mediate net flux down a
concentration gradient, the normal physiological situation would promote urea movement from blood into the rumen. In Ussing chambers in which isolated tissue is mounted, leak pathways are inevitably introduced most notably from edge damage. It is likely that in vivo, the magnitude of the diffusive mediated cellular pathway will exceed that of the paracellular pathway. Further work is required to investigate the nature of additional, phloretin-insensitive urea pathway(s) in bovine rumen and to define the direction of urea transport in vivo.

It should be noted that since there is continual loss of rumen contents to the omasum and abomasum, regeneration of rumen microflora is from a loosely adherent flora on rumen papillae. Thus delivery of urea across the rumen epithelium may play an essential role in maintaining rumen function. Potentially, UT-B is an important regulator of ruminal bacterial growth. It may provide a means for the host to regulate the flux of urea into the rumen and hence govern bacterial metabolism. Finally, the fact that both UT-A6 (24) and UT-B (11) have also now been detected in the human colon highlights the possible relevance of urea transporters to gastrointestinal function and the host-commensal microflora interaction in momogastric species.

In conclusion, we have determined the structure of the bovine UT-B gene, identified two bovine cDNAs encoding UT-B orthologs and shown that they are expressed in bovine rumen. Bovine UT-B is functionally similar to previously characterized UT-B transporters, and is located on plasma membranes of cells throughout the rumen. As such, it is likely to be responsible for the phloretin-sensitive urea trans-epithelial flux found across bovine rumen tissue. Bovine UT-B may play a role in mediating urea flux into the rumen as part of the UNS process.
Part of this work was presented in abstract form at the focused meeting of The Physiological Society at The University of Newcastle, July 22-23, 2004, 559P.

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REFERENCES


18. Ritzhaupt A, Wood IS, Jackson AA, Moran BJ and Shirazi-Beechey SP. Isolation of a RT-PCR fragment from human colon and sheep rumen RNA


FIGURE LEGENDS

Figure 1 – Organization and splicing of the bovine UT-B gene
The bovine UT-B gene spans 20kb and contains 10 exons. Exon width is representative of actual size and intron distances are representative of scale. The two distinct bovine UT-B isoforms are shown – variant 1 (bUT-B1) and variant 2 (bUT-B2).

Table 1 – Intron / exon boundaries for the 10 exons of the bovine UT-B gene
The first amino acid number refers to the larger transcript, bUT-B2, with the amino acid number for bUT-B1 in brackets. Intron sequences are shown in lowercase and exon sequences in uppercase. Consensus splice site nucleotides are underlined. Exon 2 is the bUT-B2 specific exon found in ruminal transcripts.

Figure 2 – Amino acid alignment of bovine UT-B isoforms with other UT-B transporters
The amino acid sequences are shown for bovine UT-B1, bovine UT-B2, mouse UT-B1 and human UT-B1. Bovine UT-B1 is a 384 amino acid protein that has 79% identity with human UT-B1 and 85% identity with mouse UT-B1. Bovine UT-B2 is a 439 amino acid protein that, except for an additional 55 amino acids at the N-
terminus, is identical to bovine UT-B1. Asterisks indicate that the same amino acid is present in all three species.

**Figure 3 – RT-PCR, Southern and Northern analysis of bovine rumen**

(A-C) RT-PCR experiments performed using different sets of specific bUT-B primers: (A) Primers 1 & 4 (expected bUT-B1 product size ~750bp), (B) Primers 1 & 2 (expected bUT-B1 product size ~1450bp) and (C) Primers 3 & 4 (expected bUT-B1 product size ~400bp) (Key: C = bUT-B1 clone AY624602 cDNA, BK = bovine kidney cDNA, BR = bovine rumen cDNA, N = negative control). (D) Southern blot of ~900 and ~1600 bp bovine ruminal RT-PCR products probed with $^{32}$P-labelled bUT-B1 probe. (E) Northern blot of kidney and rumen mRNA (3µg per lane) probed with a $^{32}$P-labelled, full-length bovine UT-B1 probe (high stringency) (Key: BK = bovine kidney mRNA, BR = bovine rumen mRNA, MK = mouse kidney mRNA).

**Figure 4 – Characterization of bovine UT-B1 urea transport**

(A) Compared to water-injected controls, expression of bovine UT-B1 (bUT-B1) cRNA in *Xenopus* oocytes produced a 4-fold increase in urea transport (P<0.001, ANOVA), while bUT-B2 produced a 2-fold increase (P<0.01, ANOVA). The addition of 500 µM phloretin significantly inhibited both bUT-B1 (P<0.01, ANOVA) and bUT-B2 (P<0.05, ANOVA) urea transport. (B) bUT-B1 induced urea transport is inhibited by phloretin and 2mM thionicotinamide (P<0.01, ANOVA) in a similar fashion to mouse UT-B1 (mUT-B1) induced urea transport. (Note – Values shown are mean +/- S.E.M., numbers above each column = n.)
Figure 5 – Western Analysis of bovine UT-B in rumen
MUTB detected 43-54 kDa UT-B signal in mouse kidney and bovine rumen. Prior incubation with immunizing peptide completely abolished mouse kidney signal and greatly reduced bovine rumen signal. (Key: MK – mouse kidney; RP – bovine rumen 200,000g pellet, containing plasma membrane proteins; RS – bovine rumen 200,000g supernatant, containing cytosolic proteins.)

Figure 6 – Immunolocalisation of bovine UT-B in rumen
(A) Standard light view of ruminal epithelium, with the 4 different layers labelled. (B) Immunofluorescent view showing MUTB staining for bUT-B (green) and a nucleic marker (red) in ruminal epithelium. UT-B staining present in stratum basale, spinosum and granulosum, but absent from stratum corneum. (B - INSET) Inhibition of bUT-B staining after the prior incubation of MUTB antibody with the immunizing peptide.

Figure 7 – Bovine ruminal trans-epithelial urea transport
(A) Graph showing [14C]-urea trans-epithelial fluxes plotted against simultaneously recorded [3H]-mannitol fluxes. Results are shown for both control conditions (filled circles) and in the presence of 1mM phloretin (open circles). The two solid lines are derived from linear regression analysis of these two different sets of data. (B) Graph representing reduction in the ratio of urea to mannitol fluxes from control to phloretin conditions (P<0.05, t-test, numbers represent n values).
Figure 1

E1 E2 E3 E4 E5 E6 E7 E8 E9 E10

Variant 1

Variant 2

19887bp
Figure 2

bUT-B2  MSGRSLIAGAAGDAYPGPLWRGPFGKKSGEAAHRVFPWINLAVQGPEEQEPEETSMDDN
bUT-B1  -----------------------------------------------MDDN
mUT-B1  -----------------------------------------------MEDSP
hUT-B1  -----------------------------------------------MEDSP
  *:*:* *

bUT-B2  TAVKLD-----QGGNQAPQGRGRRCLPKALGYITGDMKEFANWLKDQALQPVDFDVLRLG
bUT-B1  TAVKLD-----QGGNQAPQGRGRRCLPKALGYITGDMKEFANWLKDQALQPVDFDVLRLG
mUT-B1  TMVRVDSTPMRGENQVSPCQGRCPKALGYVTGDMKELANQLKVPVLFIDWLRLG
hUT-B1  TMTVRVDSTPMRGENQVSPCQGRCPKALGYVTGDMKELANQLKVPVLFIDWLRLG
  * *:*: ** ;**** *:**;********;**** *** ;**:**;**:**

bUT-B2  ISQVFVFVSNPIGILIVGLLQNPWPXALNCGTVSTLALLLSQDRSAIAGLQGY
bUT-B1  ISQVFVFVSNPIGILIVGLLQNPWPXALNCGTVSTLALLLSQDRSAIAGLQGY
mUT-B1  ISQVFVFVSNPIGILIVGLLQNPWPXALNCGTVSTLALLLSQDRSAIAGLQGY
hUT-B1  ISQVFVFVSNPIGILIVGLLQNPWPXALNCGTVSTLALLLSQDRSAIAGLQGY
  *********:**:**:**::*:**:*********;***:**:**

bUT-B2  LGAIISSPLMCLHAAAIGSLLGIIAGLSAPFEDIYAGLWGFNSSLACIAIGGMFMALT
bUT-B1  LGAIISSPLMCLHAAAIGSLLGIIAGLSAPFEDIYAGLWGFNSSLACIAIGGMFMALT
mUT-B1  LGAIISSPLMCLHAAAIGSLLGIIAGLSAPFEDIYAGLWGFNSSLACIAIGGMFMALT
hUT-B1  LGAIISSPLMCLHAAAIGSLLGIIAGLSAPFEDIYAGLWGFNSSLACIAIGGMFMALT
  * **********;::*:**:**;**::**:*:*::*:*;*: **:::**

bUT-B2  KVTYPEENRIFYLQSRKRTVQGPL
bUT-B1  KVTYPEENRIFYLQSRKRTVQGPL
mUT-B1  KVTYPEENRIFYLQSRKRTVQGPL
hUT-B1  KVTYPEENRIFYLQSRKRTVQGPL
  ********;*:.*:.*;** ;********:*::*;**:

bUT-B2  WQTHALLALCALFAYLAGMSVMGVGLPSGTWPCCLATLALFLFLLLTTKPNPNYKMPIS
bUT-B1  WQTHALLALCALFAYLAGMSVMGVGLPSGTWPCCLATLALFLFLLLTTKPNPNYKMPIS
mUT-B1  WQTHALLALCALFAYLAGMSVMGVGLPSGTWPCCLATLALFLFLLLTTKPNPNYKMPIS
hUT-B1  WQTHALLALCALFAYLAGMSVMGVGLPSGTWPCCLATLALFLFLLLTTKPNPNYKMPIS
  ********;*:.*:.*;** ;********:*::*;**:

bUT-B2  KVTYPEENRIFYLQSRKRTVQGPL
bUT-B1  KVTYPEENRIFYLQSRKRTVQGPL
mUT-B1  KVTYPEENRIFYLQSRKRTVQGPL
hUT-B1  KVTYPEENRIFYLQSRKRTVQGPL
  ****;::*:**:**;**:

Figure 6

--- Stratum basale
--- Stratum spinosum
--- Stratum granulosum
--- Stratum corneum

--- UT-B
--- nuclei marker
Figure 7
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Table 1