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Cloning and Expression of Bovine Glucose Transporter GLUT12

Peter J. Miller, Kiera A. Finucane, Megan Hughes, Feng-Qi Zhao

Lactation and Mammary Gland Biology Group, Department of Animal Science, University of Vermont, RM 219 Terrill Building, 570 Main Street, Burlington, Vermont 05405, USA

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Abstract

GLUT12 is a new member of facilitative glucose transporters. It was originally cloned from a human breast cancer cell line and its expression has been detected in rat mammary gland. Glucose transport across the plasma membrane of mammary epithelial cells is a rate-limiting factor in milk production. To examine GLUT12's expression and facilitate the study of GLUT12's potential role in supporting milk synthesis in lactating bovine mammary gland, we cloned bovine GLUT12 and examined its distribution of mRNA expression in bovine tissues. The full-length mRNA of bGLUT12 is 2423 base pairs long and is predicted to encode a protein of 621 amino acids with a molecular weight of approximately 67 kDa. The deduced amino acid sequence of bovine GLUT12 is 87% and 82% identical to the sequences of human and mouse GLUT12. The sequence of bGLUT12 contains several characteristically conserved sugar transporter family signatures. Analysis of current bovine genomic data indicates that bovine GLUT12 gene consists of five exons. The major *in vitro* transcription and translation product of bovine GLUT12 cDNA migrated at an apparent molecular weight of 41 kDa. In the presence of canine microsomal membranes, the translation product increased to 43 kDa, suggesting glycosylation. GLUT12 mRNA was found in all bovine tissues examined, but most abundant in bovine spleen and skeletal muscle, at intermediate levels in bovine kidney, testes, and mammary gland, and at lower levels in bovine liver, lung and intestine. Immunofluorescence staining showed that, in the presence of insulin, bGLUT12 is mainly distributed in the cytoplasm of the transiently transfected MAC-T bovine mammary epithelial cells.

In milk production, glucose is the primary substrate for synthesis of lactose which controls milk volume by maintenance of milk osmolarity. In a lactating cow, 3 kg of glucose is needed to produce 40 kg of milk each day. Glucose uptake by mammary epithelial cells is considered to be a rate-limiting step of milk synthesis (Mepham 1993). Glucose uptake by other tissues is also critical in maintaining glucose homeostasis in the lactating animal (Bell and Bauman 1997). Therefore, understanding the mechanisms and regulation of glucose uptake in the mammary gland and other tissues is a prerequisite for increasing the efficiency of energy utilization and milk production.

Glucose uptake in mammalian cells is mediated by glucose transporters. In most tissues, glucose is taken up by facilitative glucose transporters (solute carriers SLC2A, protein symbol GLUT), which mediate a bidirectional and energy-independent process of glucose transport. Thirteen members of the GLUT have been identified (Joost and Thoren 2001). Each member has different transport kinetic characteristics, substrate specificities, tissue distribution, and regulation mechanisms, indicating that each GLUT plays a distinct role in glucose utilization in different tissues. So far, only GLUT1 (GenBank accession No. NM_174602), GLUT3 (NM_174603), GLUT4 (NM_174604), and GLUT8 (NM_201528) have been cloned in bovine tissues.

GLUT12 is a recently described GLUT isoform with a preferential substrate specificity for glucose over other hexoses (Rogers et al. 2002, 2003). In normal human adult tissues, GLUT12 expression appears to be restricted mainly to insulin-sensitive skeletal

Abbreviations: bGLUT12 = bovine solute carrier family 2 member 12 (gene symbol *SLC2A12*), EST = expressed sequence tag, kb = kilobases, poly(A)$^+$ = polyadenylated RNA, RACE = rapid amplification of cDNA ends, RT-PCR = reverse transcription-polymerase chain reaction, TM = transmembrane domain, UTR = untranslated region. The nucleotide sequence data reported in this paper have been submitted to GenBank with assigned accession number AY514443.

Correspondence to: Feng-Qi Zhao; E-mail: fzhao@uvm.edu

Table 1. Sequences of oligonucleotide primers used for PCR and RACE

Primer Name	*Type*	*Sequences*
bGLUT12-F3	Forward	5′-CATCACCGGGAGGAATTAGT-3′
bGLUT12-1510F	Forward	5′-ACTGAATACCGGATCGTCAC-3′
FLAG-bGLUT12-F	Forward	5′-GGAATTCCACCATGGATTACAAGGATGACGAC GATAAGATGGTACCTGTTGAAAACGCAGAG-3′
bGLUT12-R2	Reverse	5′-AGGCCAATGAGATCCGTCAC-3′
bGLUT12-R3	Reverse	5′-AAGCCGGAACAGGCCTCTAT-3′
bGLUT12-R4	Reverse	5′-AAGGCTCCTCTGTGAAGAAG-3′

muscle and heart and is, therefore, postulated to be a second insulin-responsive glucose transporter along with GLUT4 (Rogers et al. 2002). GLUT12 was originally cloned from the human breast cancer cell line MCF-7. Its expression was also detected in rat mammary gland, where GLUT12 protein was observed in the cytoplasm of mammary epithelial cells during pregnancy and in both the cytoplasm and at the apical plasma membrane during lactation (Macheda et al. 2003). This points to a potential role for GLUT12 in transporting glucose between the cytosol of mammary epithelia and the alveolar lumen.

Expression of GLUT12 in rat mammary gland raised the possibility that GLUT12 may also play a role in the bovine mammary gland during lactation. To examine this possibility and facilitate the study of GLUT12 function in other bovine tissues in supporting lactation, we have cloned bovine GLUT12 and examined its distribution of mRNA expression in bovine tissues. We show here that GLUT12 is indeed expressed in bovine mammary gland and is localized intracellularly in the cells of bovine mammary epithelial cell line MAC-T.

Materials and methods

Animals, tissues, and RNA isolation. Tissue collections of the mammary gland, liver, kidney, lung, spleen, jejunal epithelia, and skeletal muscle from two lactating Holstein cows (*Bos taurus*) and testes from one Holstein bull as well as the RNA isolation from these samples were carried out as described previously (Zhao et al. 2005).

RACE and cloning of bGLUT12. The sequences of all primer oligonucleotides used in this study are listed in Table 1. Cloning of bovine GLUT12 was carried out using a RACE strategy. The 3′ and 5′ sequences of bGLUT12 were obtained by RACE using SMART™ RACE cDNA Amplification kits (Clontech, Palo Alto, CA). The 3′ and 5′ RACE-ready first-strand cDNAs were synthesized using 1 μg of poly(A)$^+$ RNA from bovine mammary gland. The 3′ sequence of bGLUT12 was first amplified from the 3′ RACE library using the provided universal primer (UPM, Clontech) and the forward gene-specific primer bGLUT12-1510F, which was designed from the bovine GLUT12 EST sequence (AW657233; 90% identity to human GLUT12 cDNA) and then reamplified using the provided nested universal primer (NUP, Clontech) and another forward gene-specific primer bGLUT12-F3 designed from the same EST sequence. The resulting PCR products were gel-purified, cloned into pCR4-TOPO® vector (Invitrogen, Carlsbad, CA), and sequenced using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA). The sequence of 3′ RACE products was verified in at least three independent clones.

Bovine full-length cDNA of GLUT12 was first amplified from the 5′ RACE-ready first-strand cDNA by PCR using Herculase-enhanced DNA polymerase (Stratagene, La Jolla, CA) with primers NUP and bGLUT12-R3, and then reamplified using primers NUP and bGLUT12-R4. Both bGLUT12-R3 and bGLUT12-R4 were designed from the 3′-UTR of bGLUT12 based on our RACE results. The PCR products were gel-purified and cloned into pCR2.1 (Invitrogen) to form bGLUT12/pCR2.1. The full-length bGLUT12 cDNA was sequenced, verified in at least three independent clones, and submitted to GenBank with accession No. AY514443.

DNA sequence analysis. The analysis of cDNA sequences was conducted using the computer programs of DNAstar (DNASTAR, Madison, WI), the National Center for Biotechnology Information (NCBI) BLAST site (http://www.ncbi.nlm.nih.gov/BLAST/), and the SWISS-PROT Scan-Prosite program (http://us.expasy.org/tools/scanprosite/). The multiple sequence alignment was performed with CLUSTAL W (open gap cost 10) (Thompson et al. 1994). The hydropathy plots of bGLUT12 and bGLUT1 were analyzed according to the algorithm of Kyte and Doolittle (1982) using the ProtScale program on the ExPASy proteomics server (http://us.expasy.org/cgi-bin/protscale.pl), using a 19-amino-acid window.

In vitro transcription/translation. The cDNA of bGLUT12 was excised from the plasmid bGLUT12/pCR2.1 and subcloned into a mammalian expression vector plasmid pcDNA3.1(+) (Invitrogen) to form plasmid bGLUT12/pcDNA3.1. This plasmid was transcribed by T7 polymerase and translated in the presence of L-[^{35}S]methionine (Amersham, Piscataway, NJ) using the TNT® Coupled Reticulocyte Lysate System (Promega, Madison, WI). Reactions were performed in the presence or absence of canine microsomal membranes (Promega, Madison, WI). The translation products were resolved by 12% (w/v) SDS-polyacrylamide gel electrophoresis with the addition of 3 M urea to the gel and loading buffer to reduce membrane protein aggregation, then imaged using a phosphor-capture screen and Quantity One software on a Molecular Imager FX (BIO-RAD, Hercules, CA).

RT-PCR. The expression of GLUT12 mRNA in bovine tissues was analyzed by RT-PCR as described previously (Zhao et al. 2005). The primers used for amplification of bGLUT12 were bGLUT12-1510F and bGLUT12-R2 (Table 1).

Cell culture and transient transfection. A FLAG epitope-tagged bGLUT12 construct was built by amplifying the full reading frame of bGLUT12 cDNA by PCR using pfu polymerase (Stratagene), the 3′ primer bGLUT12-R4, and the 5′ primer FLAG-bGLUT12-F (Table 1) which includes a consensus Kozak translation initiation sequence, a FLAG epitope tag, and the bGLUT12-specific primer at the putative translation initiation site. The PCR products were gel-purified, polished by *Taq* polymerase, and cloned into pCR2.1. The FLAG-tagged bGLUT12 cDNA was then excised from the pCR2.1 vector and subcloned into the mammalian expression vector pcDNA3.1 (-), which harbors a simian virus 40 origin, a cytomegalovirus promoter, and a polyadenylation site, to form plasmid FLAG-bGLUT12/pcDNA3.1.

Bovine mammary gland epithelial cell line MAC-T cells (Nexia Biotechnologies, Quebec, Canada) were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, and 5 μg/ml insulin (Huynh et al. 1991). Transfection of MAC-T cells with the FLAG-bGLUT12/pcDNA3.1, bGLUT12/pcDNA3.1, or pcDNA3.1 plasmid DNA was performed with FuGene 6 (Roche, Indianapolis, IN) following the manufacturer's instructions.

Immunofluorescent staining. MAC-T cells grown on 12-mm glass coverslips were transfected with 0.2 μg of FLAG-GLUT12/pcDNA3.1, GLUT12/pcDNA3.1, or control vector pcDNA3.1. Forty-eight hours after transfection, cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and then were permeabilized with 0.1% Triton X in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 7 \cdot H_2O$, 1.4 mM KH_2PO_4, pH 7.3) at room temperature for 15 min. Nonspecific binding sites in cells were blocked with 10% donkey serum. Primary and secondary antibodies were diluted to 2 μg/ml and 10 μg/ml in PBS containing 1% of bovine serum albumin (BSA), respectively. The cells were incubated with mouse anti-FLAG M2 monoclonal antibody (Stratagene, La Jolla, CA) overnight at 4°C with high humidity. Cells were rinsed with PBS and incubated in the dark with shaking at room temperature for 1 h with the Alexa Fluor 647 anti-mouse secondary antibody (Molecular Probes, Eugene, OR). The nuclear counterstain Sytox (Molecular Probes) was used at a dilution of 1:10000 in PBS. Coverslips were mounted using Aqua Poly/Mount (Polysciences, Warrington, PA). Cell images were captured using Bio-Rad MRC 1024ES Confocal Laser Scanning Imaging System with LaserSharp2000 software (Bio-Rad, Hercules, CA).

Results and discussion

Cloning of bovine GLUT12 cDNA by RACE. A RACE strategy was adapted to obtain the full-length cDNA sequence of bovine GLUT12 from bovine mammary gland (Fig. 1). The full-length mRNA of bGLUT12 is 2423 bp long (GenBank accession No. AY514443). Open reading frame analysis suggests bovine GLUT12 is composed of 621 amino acids, with a molecular weight of approximately 67 kDa.

The deduced amino acid sequence of bovine GLUT12 is 20%, 21%, 20%, and 21% identical to or 38%, 38%, 38%, and 37% conserved with those of bovine GLUT1 (GenBank accession No. NP_777027), GLUT3 (NP_777028), GLUT4 (NP_777029), and GLUT8 (NP_963286), respectively (Fig. 2A). The major structural differences unique to bGLUT12 are a longer loop between the putative transmembrane domains (TM) 9 and 10 (see below) and longer amino- (N-) and carboxyl- (C-) termini (Fig. 2A).

The deduced amino acid sequence of bovine GLUT12 is 87% and 82% identical to or 92% and 90% conserved with the sequences of human (NP_660159) and mouse (NP_849265) GLUT12, respectively (Fig. 2B). The major differences among species are also located in the most divergent regions among GLUTs.

Sequence analysis of bovine GLUT12. The hydropathy plot analysis of bovine GLUT12 by ProtScale (Fig. 3) revealed that the distribution

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bp                                                                                  AA
-90                                                         atgcaactatgggaa
-75    aagtgaccgctcgtgtcagttggggaagtcggggtggtttggtgcccagggaacggctgtaacttctacgtgacc
1      ATGGTACCTGTTGAAAACGCAGAGGGCCCCAGTCTGCTGAAACCGAAGGGGCGAGCGGCGGAGACTGATGGCAGC
        M  V  P  V  E  N  A  E  G  P  S  L  L  K  P  K  G  R  A  A  E  T  D  G  S    25
76     GACAGAGCCAGCGGCGGCCCGCATCCTCCCTGGGCGCGAGGTTGTGGCATGTATACTCTCCTGTCGTCTGTCACA
        D  R  A  S  G  G  P  H  P  P  W  A  R  G  C  G  M  Y  T  L  L  S  S  V  T    50
151    GCTGCTGTCAGTGGCTTCCTGGTGGGGTATGAACTTGGAATCATCTCTGGGGCTCTTCTGCAAATAAGAACCTTG
        A  A  V  S  G  F  L  V  G  Y  E  L  G  I  I  S  G  A  L  L  Q  I  R  T  L    75
226    TTAGTCCTGACCTGCCATGAGCAGGAAATGGTGGTGAGCTCCCTGCTTATCGGGGCCCTCCTGGCCTCTCTAATC
        L  V  L  T  C  H  E  Q  E  M  V  V  S  S  L  L  I  G  A  L  L  A  S  L  I    100
301    GGAGGAGTCCTGATTGACAGGTACGGGAGAAGGGCCGCCATCATTTTGTCCTCCTGCCTCCTCGGACTTGGAAGC
        G  G  V  L  I  D  R  Y  G  R  R  A  A  I  I  L  S  S  C  L  L  G  L  G  S    125
376    CTGGTCTTGATTATCAGTTTATCTTACACCACTCTTATAGGGGGGCGCATCGCTATTGGAGTTTTCATTTCACTC
        L  V  L  I  I  S  L  S  Y  T  T  L  I  G  G  R  I  A  I  G  V  F  I  S  L    150
451    TCCTCCACCGCCACTTGTGTCTACATCGCAGAAATTGCTCCCCAACACAGAAGAGGCCTTCTCGTGTCACTGAAT
        S  S  T  A  T  C  V  Y  I  A  E  I  A  P  Q  H  R  R  G  L  L  V  S  L  N    175
526    GAACTGATGATTGTCATCGGCATTCTTTTTGCCTACATTTCAAATTATGCATTTGCCAACATTTCCCATGGCTGG
        E  L  M  I  V  I  G  I  L  F  A  Y  I  S  N  Y  A  F  A  N  I  S  H  G  W    200
601    AAATACATGTTTGGCCTTGTTATTCCCTTGGGAGTTTTGCAAGCCATTGCAATGTACTTTCTTCCTCCAAGTCCT
        K  Y  M  F  G  L  V  I  P  L  G  V  L  Q  A  I  A  M  Y  F  L  P  P  S  P    225
676    CGATTTCTGGTGATGAAGGGACATGAGGAAGCTGCTAGTAAGGTTCTTGGAAAGCTGAGAGCTGTCTTGGATACA
        R  F  L  V  M  K  G  H  E  E  A  A  S  K  V  L  G  K  L  R  A  V  L  D  T    250
751    ACAGAGGAACTCACGGTGATAAAGTCTTCCCTGAAAGATGAATATCAGTACAGTTTTTGGGATCTGTTTCGTTCA
        T  E  E  L  T  V  I  K  S  S  L  K  D  E  Y  Q  Y  S  F  W  D  L  F  R  S    275
826    AAAGACAACATGAGGACCCGAATCATGATAGGCTTAACGCTGGTATTTTTTGTACAGATCACTGGCCAGCCAAAC
        K  D  N  M  R  T  R  I  M  I  G  L  T  L  V  F  F  V  Q  I  T  G  Q  P  N    300
901    ATATTATTCTATGCATCTACTGTTTTGAAGTCTGTTGGCTTCCAAAGCAATGAGGCAGCTAGCCTCGCCTCCACC
        I  L  F  Y  A  S  T  V  L  K  S  V  G  F  Q  S  N  E  A  A  S  L  A  S  T    325
976    GGGGTTGGGGTCGTCAAGGTCATCAGCACCATCCCAGCCACCCTTCTCGTAGACCAGGTTGGGAGCAAAACCTTC
        G  V  G  V  V  K  V  I  S  T  I  P  A  T  L  L  V  D  Q  V  G  S  K  T  F    350
1051   CTGTGTATCGGTTCCTCTGTGATGGCAGCTTCGTTGGTGACCATGGGCATCGTGAATCTCAACATCCACATGAAC
        L  C  I  G  S  S  V  M  A  A  S  L  V  T  M  G  I  V  N  L  N  I  H  M  N    375
1126   TTCACCAGTATCTGCAGAAACCATAGTCCTATCAACCAGTCCTTGGATGAGTCTGTGTTTTATGGACCAGGTAAC
        F  T  S  I  C  R  N  H  S  P  I  N  Q  S  L  D  E  S  V  F  Y  G  P  G  N    400
1201   TTGTCAGCCAGCAATGACACTCTTAGAGAATCCTTTAAAGGGATGACTTTCCACAGCAGAAGCTCACTGAGGCCC
        L  S  A  S  N  D  T  L  R  E  S  F  K  G  M  T  F  H  S  R  S  S  L  R  P    425
1276   ACGAGAAATGACATAAATGGGAGAGGAGAAACGACCCTGGCATCCTTACCAAATGCTGGACTAAGCCAAACTGAA
        T  R  N  D  I  N  G  R  G  E  T  T  L  A  S  L  P  N  A  G  L  S  Q  T  E    450
1351   TACCAGATCGTCACAGACTCTGCAGATGTCCCCACTTTTTTGAAATGGCTTTCTTTAGCCAGCTTGCTTGTTTAC
        Y  Q  I  V  T  D  S  A  D  V  P  T  F  L  K  W  L  S  L  A  S  L  L  V  Y    475
1426   GTTGCTGCCTTTTCAATTGGTCTAGGACCAATGCCCTGGTTGGTGCTGAGCGAGATTTTTCCCGGAGGAATTCGA
        V  A  A  F  S  I  G  L  G  P  M  P  W  L  V  L  S  E  I  F  P  G  G  I  R    500
1501   GGACGTGCCATGGCTCTAACTTCCAGCATGAACTGGGGCATCAATCTCCTCATCTCTCTGACATTTTTGACGGTG
        G  R  A  M  A  L  T  S  S  M  N  W  G  I  N  L  L  I  S  L  T  F  L  T  V    525
1576   ACGGATCTCATTGGCCTGCCGTGGGTGTGCTTTATATATACAGTTATGAGTCTAGCATCCCTGGTTTTTGTTATT
        T  D  L  I  G  L  P  W  V  C  F  I  Y  T  V  M  S  L  A  S  L  V  F  V  I    550
1651   GTGTTTATACCTGAAACAAAGGGATGCTCTTTGGAACAAATATCAGTGGAGCTGGCAAAAGAGAACTATGTGAAA
        V  F  I  P  E  T  K  G  C  S  L  E  Q  I  S  V  E  L  A  K  E  N  Y  V  K    575
1726   AACAACATTTGTTTTATGAGTCATCACCGGGAGGAATTAGTGCCAAAACAGCTTCAAAAAAGAAAACCCCAAGAG
        N  N  I  C  F  M  S  H  H  R  E  E  L  V  P  K  Q  L  Q  K  R  K  P  Q  E    600
1801   CAGTTCTTGGAGTCTAAAAAGCTAAGGGGTAAGGGACAGCCAAGGCAGCTTTCTTCAGAGGTCTAAtggcctcaa
        Q  F  L  E  S  K  K  L  R  G  K  G  Q  P  R  Q  L  S  S  E  V  *             621
1876   Aactcttcaagtgttgatacagaaagagtacttacggtgtcttcctatcaatatgtgttagccacgcctgtgttt
1951   tcttttcagtgtcatagagctagtttacaaaggataccctgaaattatagtctttactcattaccccaccccca
2026   aaggagccatgaaaggtatttgaggaacaaggtcccaagtggtctctttcttactctgagcaggacaccaggata
2101   aaaataataccagctggttcagtattttccaccttcttcacagaggagcctttgaaagacgatgaactagtgatg
2176   agagcaacttttatcttaaggtttgttatgcatagaggcctgttgcggctttattattcagacacataagttgtc
2251   cgaacataagcttgcagttctttgcctaccaggacactccccacactaggtcttgtgcaaaaaaaaaaaaaaaaa
2326   aaaaaaaa
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Fig. 1. Full-length cDNA and deduced amino acid sequence of bovine GLUT12. In the cDNA sequence, lower-case letters represent the 5′ and 3′ UTRs and the upper-case letters represent the coding region. The predicted amino acid sequence is shown in upper-case letters beneath the coding sequence. The predicted N-glycosylation sites are in boldface. The nucleotide sequence of bovine GLUT12 mRNA has been submitted to the GenBank nucleotide database with accession No. AY514443.

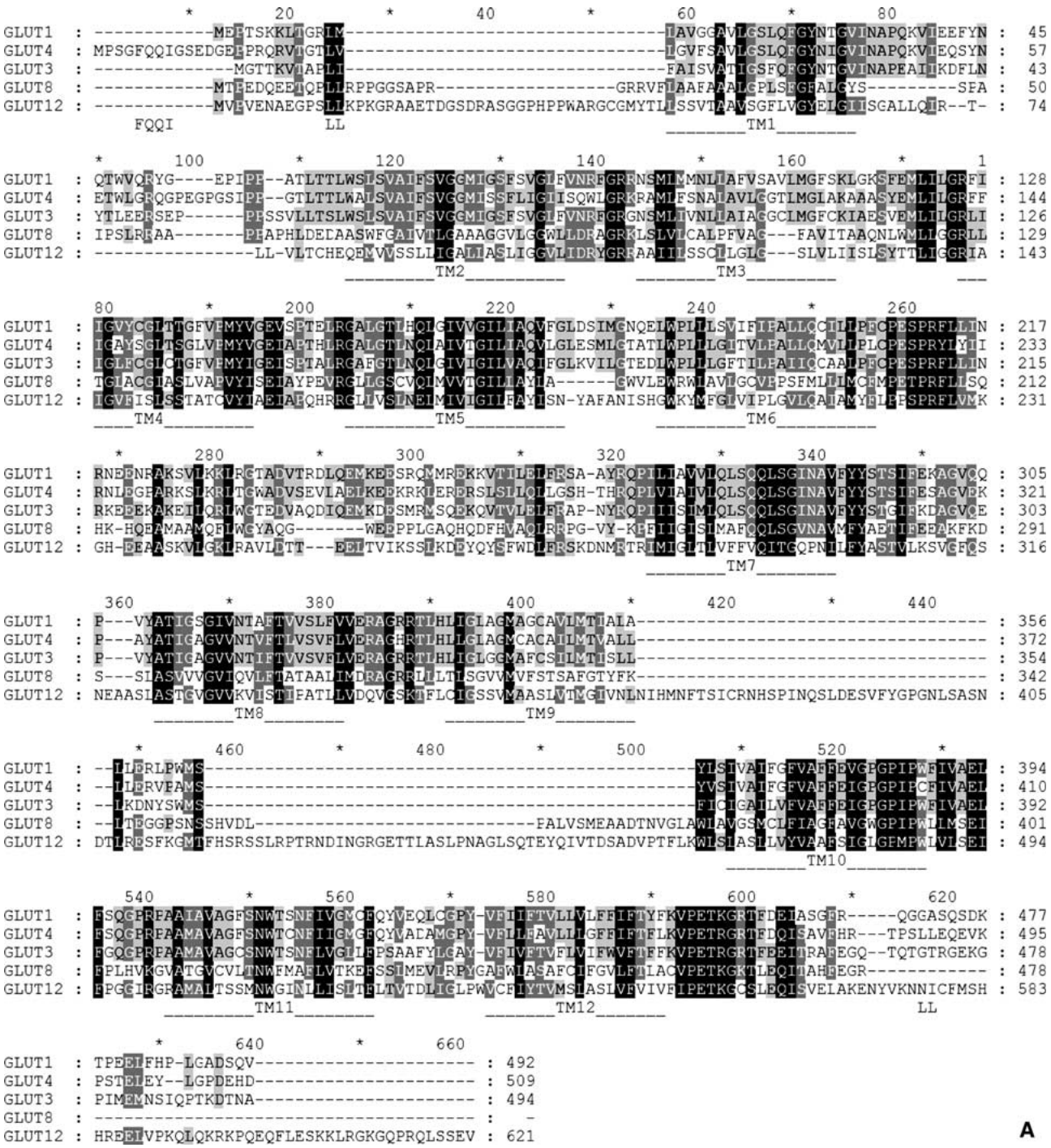

Fig. 2. (Continued on next page).

pattern of its hydrophobic and presumed membrane-spanning (TM) segments generally favors the proposed secondary structure of GLUTs, a 12-helix model (Fig. 2A) (Joost and Thorens 2001), except that the presence of TM11 is questionable. The bGLUT12 has two large exoplasmic loops between TM 6 and 7 (loop 6) and between 9 and 10 (loop 9) in addition to two large exoplasmic termini (Figs. 2A and 3). The large loop 9 contains glycosylation site(s) (see below) and is a structural characteristic of class III members of GLUT family. Therefore, the hydropathy plot of bGLUT12 is more similar to that of another class III member, bGLUT8, than to the class I member, bGLUT1 (Zhao et al. 2004).

The sequence of bGLUT12 contains several characteristically conserved sugar transporter family

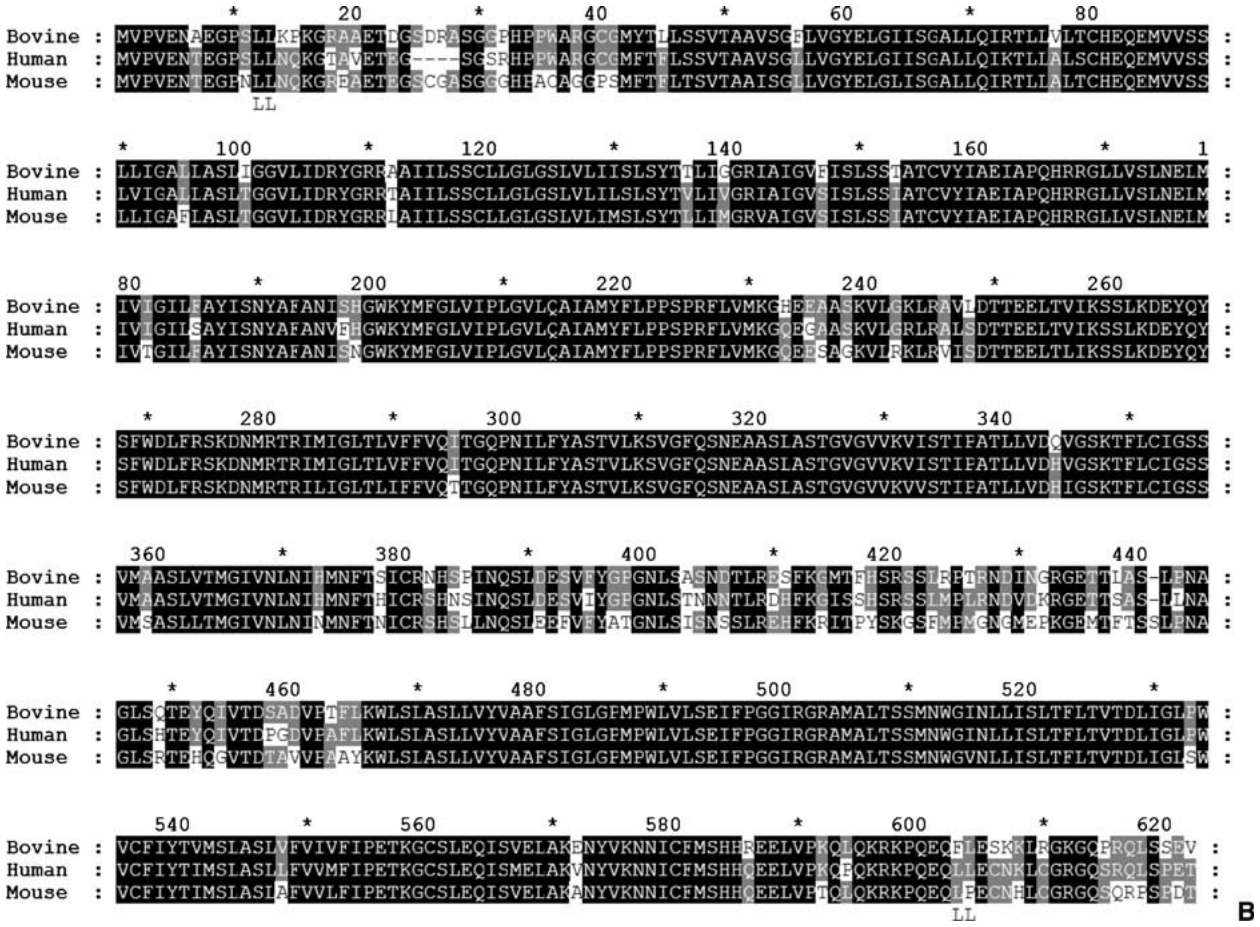

Fig. 2. (**A**) Multiple sequence alignment of the deduced amino acid sequence of bovine GLUT12 with the sequences of other known members of bovine GLUT family: bGLUT1 (GenBank accession No. NP_777027), bGLUT3 (NP_777028), bGLUT4 (NP_777029), and bGLUT8 (NP_963286). Positions of presumed membrane-spanning helices (TM) of the GLUT proteins are given by the numbered dashed lines at the bottom of the sequence alignment. (**B**) Multiple sequence alignment of the deduced amino acid sequence of bovine GLUT12 with the sequences of human (NP_660159) and mouse (NP_849265) GLUT12. The alignments were performed with the CLUSTAL W program (open gap cost 10). Residues that are highlighted by black shading background represent absolutely conserved amino acids and the gray shading indicates three (**A**), two (**B**), or more conserved residue at that position. The NH_2- and COOH-terminal dileucine and FQQI motifs described in text are annotated.

signatures. The sugar transport proteins signature 1 (SUGAR_TRANSPORT_1) (accession No. PS00216) is located between amino acids 101 and 118 (GGVLIDrYGRRaaiilsS). The sequence between amino acids 47 and 557 in bGLUT12 matches with major facilitator superfamily (MFS) profile (PS50850). In addition, bGLUT12 contains other motifs that may be critical for either transport activity or substrate specificity (Joost and Thoren 2001): GRK/R in loop 2, GR in loop 3, E···RG in loop 4, PXXPR in loop 6, GXGPXXW in helix 10, and PETKG in the C-terminal tail.

Scan-Prosite analysis predicted five potential N-glycosylation sites (PS00001) positioned at amino acids 195–198 (NISH), 375–378 (NFTS), 387–390 (NQSL), 400–403 (NLSA), and 405–408 (NDTL) of bGLUT12 (Fig. 1). The last four sites are all located in the putative loop 9 which is a structural characteristic of class III members of GLUT family, rather than in loop 1 as class 1 GLUTs. The glycosylation of bGLUT12 is confirmed in our *in vitro* translation study below. However, which of these predicted sites or whether all can be glycosylated needs further investigation. In addition, numerous protein kinase C (PS00005) and casein kinase II (PS00006) phosphorylation sites, several N-myristoylation sites (PS00008), and one amidation site (PS00009) are also predicted with a high probability of occurrence but the functional significance of these sites remains to be investigated.

Interestingly, Scan-Prosite analysis also predicted a bipartite nuclear targeting sequence

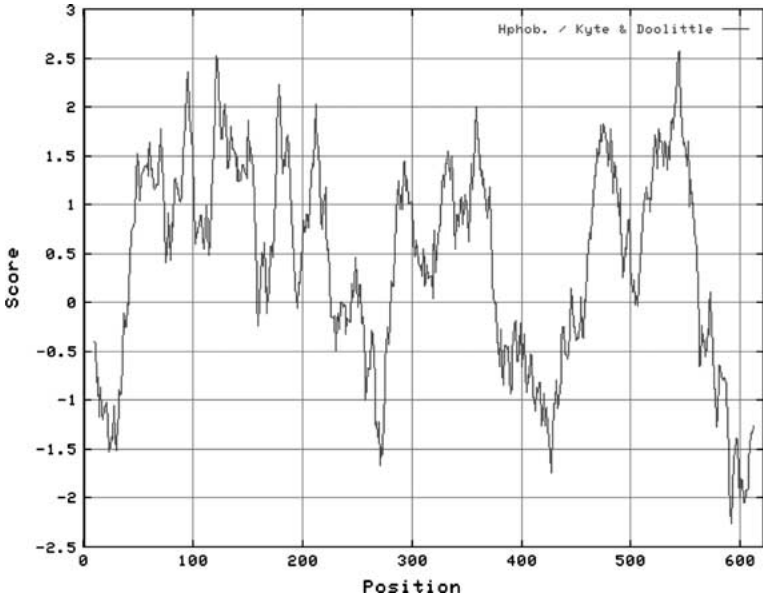

Fig. 3. Hydropathy plot of bovine GLUT12. The hydropathy plot has been derived according to the algorithm of Kyte and Doolittle (1982), using a 19-amino-acid window and the linear weight variation model.

(KRkpqeqfleskklrgk; PS00015) at amino acids 595–611 of bGLUT12. The bipartite nuclear targeting sequence is a structural signal that specifies selective accumulation of a protein in the nucleus. About 56% of known nuclear proteins contain the bipartite nuclear targeting sequence (Dingwall and Laskey 1991). The significance of existence of this sequence in bGLUT12 is not known since bGLUT12 is a glucose carrier and was detected in the cytoplasm of MAC-T bovine mammary epithelial cells (see below). Similar to bGLUT8, bovine GLUT12 has a dileucine motif in the N-terminus, at a similar position to the GLUT4 FQQI motif (Fig. 2A). Both dileucine and FQQI motifs have been shown to be required to target the protein to intracellular compartments (Ibberson et al. 2000; Khan et al. 2004; Lisinski et al. 2001; Uldry et al. 2001). However, unlike GLUT4 and human GLUT12, bGLUT12 does not have a dileucine motif at the C-terminal region (Fig. 2A and B).

Genomic organization of bovine GLUT12. To determine the structure of the bovine GLUT12 gene, we BLAST searched the recently released bovine genomic database (http://www.hgsc.bcm.tmc.edu/blast/?organism=Btaurus) using bGLUT12 cDNA. The search results indicated that the bovine GLUT12 gene structure is very similar to human GLUT12 and is composed of 5 exons ranging from 123 to 1341 bp (Table 2 and Fig. 4). The bovine GLUT12 gene spans 36 kb and the intron sizes range from 4011 to 10,797 bp. Both the 5′ and 3′ splice

Table 2. Nucleotide sequence of intron-exon junction of the bovine GLUT12 gene in comparison with human GLUT12 gene[a]

					Size (bp)	
Exon #	*Species*	*3′* intron	*Exon sequence*	*5′* intron	*Exon*	*Intron*
1	Bovine	N/A	ATG CAA CTA ..... GGG CGC GAG	gtaaagcgag	205[b]	7628
	Human	N/A	ACA TTA GCT ..... GGG CGA GAG	gtaaagtgag	236[c]	22656
2	Bovine	ctgctcgtag	GTT GTG GCA ..... TAG GAC CAA	gtaagtattt	1341	10797
	Human	ctgcttgcag	GCT GCG GCA ..... TAG GAC CAA	gtaagtactt	1341	21446
3	Bovine	tttcttccag	TGC CCT GGT ..... CGG TGA CGG	gtaagaactt	123	4011
	Human	tttcttctag	TGC CCT GGC ..... CTG TAA CTG	gtaagaagtc	123	4682
4	Bovine	tctcccccag	ATC TCA TTG ..... GGC AAA AGA	gtgagtattg	133	11469
	Human	tcctttatag	ATC TTA TTG ..... AGC AAA AGT	gtaagtatta	133	10688
5	Bovine[d]	tctctttcag	GAA CTA TGT ..... TGC AAA AAA	N/A	602	N/A
	Human	tctctttcag	GAA CTA TGT ..... AAA TAA TTC	N/A	815[c]	N/A

[a]The sizes of exons and introns of human GLUT12 gene were based on the human Chromosome 6 sequence in GenBank (accession No. NC_000006) and human GLUT12 cDNA (NM_145176). The sizes of exons and introns of bovine GLUT12 gene were based on our bovine GLUT12 cDNA and the sequences of bovine genomic contigs (NW_622151.1) in GenBank (*Bos taurus* Genome Assembly 2004 10 06).
[b]Exact size of exon 1 was based on our 5′ RACE data.
[c]Approximate size of this exon was based on human GLUT12 sequence (NM_145175).

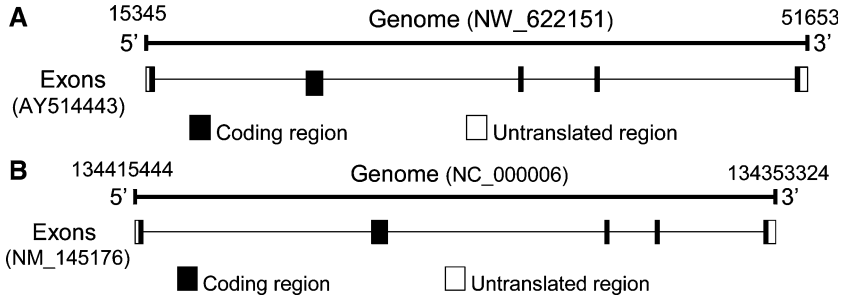

Fig. 4. Genomic organization of bovine (**A**) and human (**B**) GLUT12 genes. (**A**) The figure was drawn based on the bovine genome contig BtUn_WGA456984 sequence (accession No. NW_622151.1), bovine GLUT12 cDNA (AY514443), and its deduced amino acid sequence (NP_001011683). (**B**) The figure was drawn based on the human Chromosome 6 sequence (accession No. NC_000006), human GLUT12 cDNA (NM_145176) and its deduced amino acid sequence (NP_660159).

junctions and the sizes of each exon are listed in Table 2. In each case, the sequences at the boundaries complied with the canonical AG..GT acceptor/donor splice sites. By comparison, the human GLUT12 gene spans 62 kb and the intron sizes range from 4682 to 22,656 bp (Fig. 4). Human GLUT12 cDNA (NM_145176) completely aligned to only one genomic sequence (NT_000006) of Chromosome 6 (6q23.2), indicating that the human genome has only one copy of the GLUT12 gene. It is not clear whether bovine also has only one copy of GLUT12 gene in its genome and on which chromosome it is located.

In vitro transcription and translation of bGLUT12 cDNA and glycosylation of bGLUT12. Coupled *in vitro* transcription and translation were used to characterize the bGLUT12 gene product (Fig. 5). The translation product migrated at an apparent molecular weight of 41 kDa. On addition of canine microsomal membranes to the translation reactions, the apparent size of translation product showed a small increase, indicating glycosylation of bGLUT12. The same shift was also observed in FLAG-tagged bGLUT12 and in the positive control of bGLUT1 (Zhao et al. 2004). The functional significance of the glycosylation of bGLUT12 is not known. However, it has been shown that N-glycosylation is essential for the biological activity of GLUT1 but is not required for its translocation from the intracellular membrane pool to the plasma membrane in different cells (Ahmed and Berridge 1999; Samih et al. 2000, 2003).

Distribution of GLUT12 mRNA in bovine tissues. Expression of GLUT12 mRNA was analyzed by RT-PCR in the mammary gland, liver, kidney, lung, spleen, intestine, and skeletal muscle of two lactating cows and in testicular tissue of one bull using specific primers derived from the bGLUT12 cDNA (Fig. 6). GLUT12 mRNA was found in all bovine tissues examined, but most abundantly in bovine spleen and skeletal muscle, at intermediate levels in bovine kidney, testes, and mammary gland, and at lower levels in bovine liver, lung, and intestine. The relative expression levels of GLUT12 mRNA in bovine tissues are not completely consistent with its distribution reported in human, where GLUT12 mRNA is found most abundantly in insulin-sensitive tissues (heart and skeletal muscle) and at very low levels in spleen and kidney (Rogers et al. 2002). The different expression patterns of GLUT12 in spleen and kidney between two species may represent species differences or may be a result of expression changes at the different developmental stages. Based on this distribution pattern in the human, it was proposed that GLUT12 may be another insulin-sensitive GLUT. Our bovine data do not support this idea, at least in bovine species.

Subcellular localization of bGLUT12 in transfected MAC-T cells. Since bovine GLUT12 antibodies are not yet available, we generated a

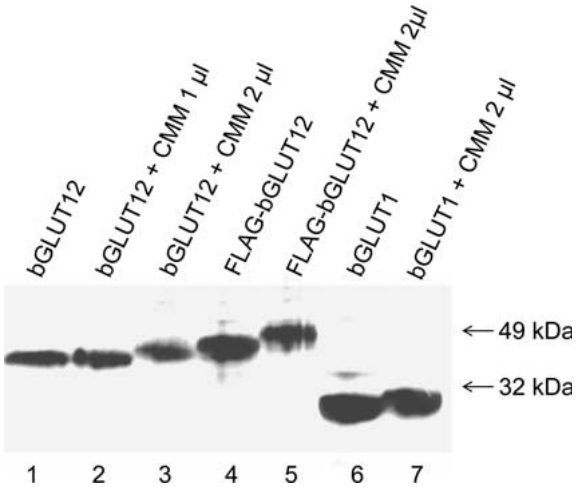

Fig. 5. *In vitro* transcription and translation of bovine GLUT12 (bGLUT12), FLAG-tagged bGLUT12 (FLAG-bGLUT12), and bGLUT1 cDNAs with or without addition of canine microsomal membranes (CMM) in the reaction. The positions of two protein size markers are indicated (kDa = kilodaltons).

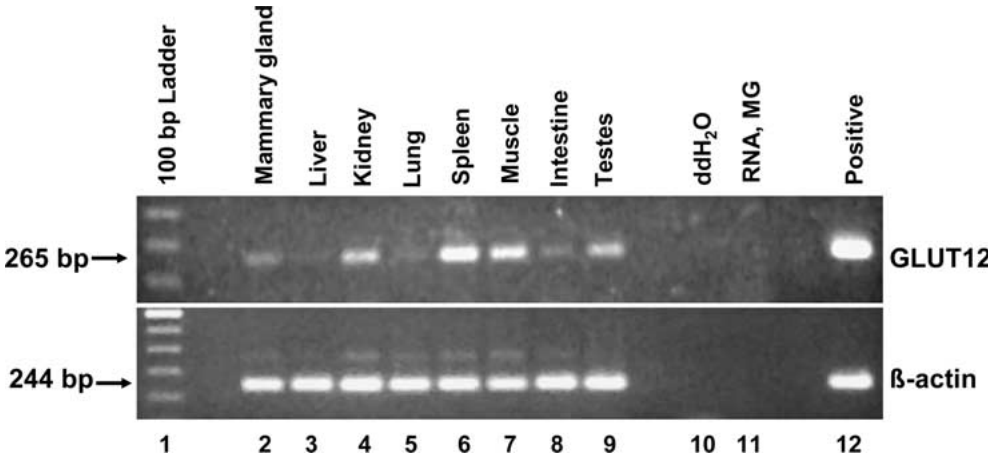

Fig. 6. Tissue distribution of GLUT12 mRNA in bovine by RT-PCR. The first-strand cDNAs were synthesized from 5 μg of total RNA (pooled from two lactating cows) isolated from the mammary gland (lane 2), liver (3), kidney (4), lung (5), spleen (6), skeletal muscle (7), and small intestinal mucosa (8), or from 5 μg of total RNA isolated from testes (9) of one bull. The cDNAs, along with ddH_2O, RNA from the mammary gland (MG) (10 and 11, negative controls), and 1 ng of bGLUT12/pCR2.1 plasmid DNA or pTRI-*β*-Actin-Mouse Antisense Control Template (12, positive controls) were amplified using primers for bGLUT12 (upper panel) or ß-actin (bottom panel). The sizes of RT-PCR products are indicated by arrows.

FLAG epitope-tagged bGLUT12 construct as described in the *Materials and methods* section. This construct was transiently transfected into cells of the bovine mammary gland cell line MAC-T, in the presence of insulin in the culture medium. Indirect immunofluorescent staining of these transfected cells using an anti-FLAG antibody was carried out to visualize the subcellular localization of the ectopically expressed bGLUT12. As shown in Fig. 7, FLAG-tagged bGLUT12 appeared to be distributed to the cytoplasm of insulin-treated cells (Fig. 7A). No signal was detected in the primary antibody-omitted

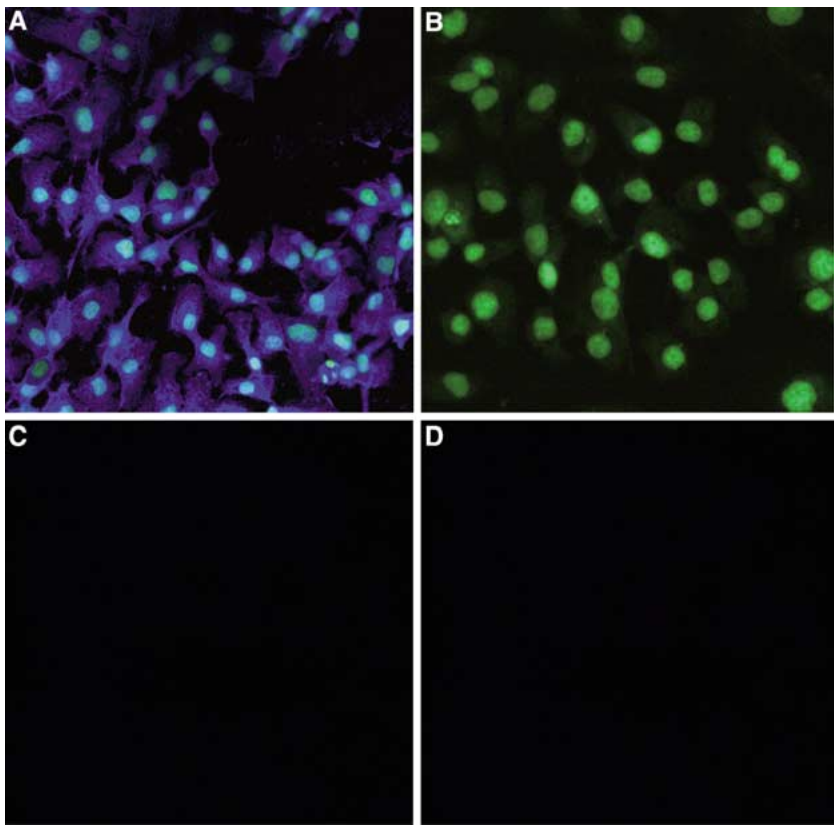

Fig. 7. Localization of bovine GLUT12 in bovine mammary gland cell line MAC-T cells. MAC-T cells were transiently transfected with a FLAG epitope-tagged bGLUT12 construct FLAG-bGLUT12/pcDNA3.1 (**A, B**), a nontagged construct bGLUT12/pcDNA3.1 (**C**), or the control vector pcDNA3.1 (**D**). After transient expression, cells were fixed, permeabilized, and incubated with (**A, C, D**) or without (**B**) a mouse monoclonal antibody directed against the FLAG epitope. Cells were then incubated with Alexa Fluor 647 conjugated donkey anti-mouse second antibody. Cells in A and B were counterstained with Sytox nuclear stain. The images were captured by a laser scanning confocal microscope (40×).

control experiment (Fig. 7B) and in the cells transfected with a nontagged bGLUT12 construct (Fig. 7C) or with the control vector plasmid (Fig. 7D).

The intracellular localization of bGLUT12 is consistent with presence of a dileucine motif in the N-terminus of bGLUT12, at a similar position to the GLUT4 FQQI motif (Fig. 2A), similar to bGLUT8 (Zhao et al. 2004). Both dileucine and FQQI motifs have been shown to be required to target the protein to intracellular compartments (Ibberson et al. 2000; Khan et al. 2004; Lisinski et al. 2001; Uldry et al. 2001). However, unlike GLUT4 and human GLUT12, bGLUT12 does not have a dileucine motif at the C-terminal region (Fig. 2A and B). The significance of the absence of the dileucine motif at the C-terminal region is not known. It is well established that GLUT4 can be translocated to the plasma membrane under insulin stimulation (Watson et al. 2004). Although our immunofluorescent staining showed an intracellular localization of GLUT12 in the MAC-T mammary gland cell under insulin condition, it can not rule out the possibility that other hormones, such as the lactogenic hormone prolactin, may induce its translocation from the cytoplasmic location to the plasma membrane. It has been shown that in rat mammary gland, GLUT12 protein is located in the cytoplasm of mammary epithelial cells during pregnancy but in both the cytoplasm and at the apical plasma membrane during lactation (Macheda et al. 2003).

Conclusions

In this article we report the cDNA and the deduced amino acid sequences of bovine GLUT12 and its expression in bovine tissues. Our data lay the groundwork for future studies aimed at unraveling the functional roles of GLUT12 in supporting milk production and maintaining glucose homeostasis during lactation.

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References

1. Ahmed N, Berridge MV (1999) N-glycosylation of glucose transporter-1 (Glut-1) is associated with increased transporter affinity for glucose in human leukemic cells. Leuk Res 23, 395–401
2. Bell AW, Bauman DE (1997) Adaptations of glucose metabolism during pregnancy and lactation. J Mammary Gland Biol Neoplasia 2, 265–278
3. Dingwall C, Laskey RA (1991) Nuclear targeting sequences—a consensus. Trends Biochem Sci 16, 478–481
4. Huynh HT, Robitaille G, Turner JD (1991) Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp Cell Res 197, 191–199
5. Ibberson M, Uldry M, Thorens B (2000) GLUTX1, a novel mammalian glucose transporter expressed in the central nervous system and insulin-sensitive tissues. J Biol Chem 275, 4607–4612
6. Joost JG, Thorens B (2001) The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). Mol Membr Biol 18, 247–256
7. Khan AH, Capilla E, Hou JC, Watson RT, Smith JR, et al. (2004) Entry of newly synthesized GLUT4 into the insulin-responsive storage compartment is dependent upon both the amino terminus and the large cytoplasmic loop. J Biol Chem 279, 37505–37511
8. Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157, 105–132
9. Lisinski I, Schurmann A, Joost HG, Cushman SW, Al-Hasani H (2001) Targeting of GLUT6 (formerly GLUT9) and GLUT8 in rat adipose cells. Biochem J 358, 517–522
10. Macheda ML, Williams ED, Best JD, Wlodek ME, Rogers S (2003) Expression and localisation of GLUT1 and GLUT12 glucose transporters in the pregnant and lactating rat mammary gland. Cell Tissue Res 311, 91–97
11. Mepham TB (1993) The development of ideas on the role of glucose in regulating milk secretion. Aust J Agric Res 44, 509–522
12. Rogers S, Macheda ML, Docherty SE, Carty MD, Henderson MA, et al. (2002) Identification of a novel glucose transporter-like protein—GLUT-12. Am J Physiol Endocrinol Metab 282, E733–E738
13. Rogers S, Chandler JD, Clarke AL, Petrou S, Best JD (2003) Glucose transporter GLUT12-functional characterization in Xenopus laevis oocytes. Biochem Biophys Res Commun 308, 422–426
14. Samih N, Hovsepian S, Aouani A, Lombardo D, Fayet G (2000) Glut-1 translocation in FRTL-5 thyroid cells: role of phosphatidylinositol 3-kinase and N-glycosylation. Endocrinology 41, 4146–4155
15. Samih N, Hovsepian S, Notel F, Prorok M, Zattara-Cannoni H, et al. (2003) The impact of N- and O-glycosylation on the functions of Glut-1 transporter in human thyroid anaplastic cells. Biochim Biophys Acta 1621, 92–101
16. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22, 4673–4680

17. Uldry M, Ibberson M, Horisberger JD, Chatton JY, Riederer BM, et al. (2001) Identification of a mammalian H(+)-myo-inositol symporter expressed predominantly in the brain. EMBO J 20, 4467–4477
18. Watson RT, Kanzaki M, Pessin JE (2004) Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. Endocr Rev 25, 177–204
19. Zhao F-Q, Miller PJ, Wall EH, Zheng Y-C, Dong B, et al. (2004) Bovine glucose transporter GLUT8: cloning, expression, and developmental regulation in mammary gland. Biochim Biophys Acta 1680, 103–113
20. Zhao F-Q, Zheng Y-C, Wall EH, McFadden TB (2005) Cloning and Expression of Bovine sodium/Glucose Cotransporters. J Dairy Sci 88, 182–194