

Cloning of a bovine orphan transporter and its short splicing variant

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Abstract We have isolated a cDNA (bv7-3) encoding a member of the Na⁺,Cl[−]-dependent transporter family and its short splicing variant (bv7-3s) by screening a bovine retina cDNA library. Sequence analysis revealed that bv7-3 encodes a protein of 729 amino acids and is a bovine homologue of the rat orphan transporter v7-3-2. bv7-3s contains 265 amino acids, sharing 252 N-terminal amino acids with bv7-3. Both mRNAs for bv7-3 and bv7-3s were detected in nervous system by Northern blot analysis. In immunofluorescence analysis in transfected HEK 293T cells, myc-tagged bv7-3 was mainly detected on the plasma membrane, whereas myc-tagged bv7-3s showed a pattern of intracellular membrane staining.

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Key words: Splicing variant; Orphan transporter; Molecular cloning; Cellular localization; Bovine

1. Introduction

Cloning of the transporter for γ -aminobutyric acid [1] and norepinephrine [2] allowed the subsequent isolation of a number of cDNAs encoding homologous Na⁺,Cl[−]-dependent transporters, which has given great insight into the structural and pharmacological mechanisms of transporters [3–5]. Recently, several splicing variants of transporters have been cloned, which indicated the occurrence of splicing in transporter genes and regulation of mRNA expression. Glycine transporter 1 (GlyT1) and glycine transporter 2 (GLYT2) have several transporter variants (GlyT1a, GlyT1b, GlyT1c, GLYT2a and GLYT2b) which differ only in their N-terminal region and have similar transport affinity for glycine. Variants of GlyT1 show differential tissue distribution of mRNAs indicating the regulation of genetic elements responsible for the tissue-specific expression [6–12]. Serotonin transporter also has variants at the 5' non-coding region that cause differential expression of mRNA [13–16]. Recently, a variant of the bovine noradrenaline transporter with a different C-terminal cytoplasmic-facing region (18 amino acids) and 3' untranslated region was isolated [17]. It was revealed that the C-terminal region is important for correct targeting to the membrane and then functional expression. With the Na⁺,K⁺,OH[−]-dependent transporter family, Utsunomiya-Tate et al. described the rat liver glutamate transporter (GLT1) which has different 5' ends determining the tissue specificity of mRNA expression, and

showed that co-expression of both GLT1 with different 3' ends results in an increase in $V_{\max}$ of transport without a change in K_m [18]. Recently, Mayer et al. described two alternatively spliced transcripts of the human glutamate transporter EAAT2 (GLT1) that resulted from exon skipping or usage of internal splice sites though details as to their function and distribution have yet to be reported [19]. Thus, there have been many studies concerning a wide variety of functions of splicing variants of transporters.

Here we report a novel transporter termed bv7-3 and its splicing variant bv7-3s. They share an N-terminal region with four putative transmembrane domains. Differential subcellular localization of these clones was also shown.

2. Materials and methods

2.1. Screening and sequencing

The oligonucleotide probe (5'-TAGGGGATCAGGAAG(A/G)C-ACCTCCGCCGTTCTTGTAGCACAGGTAGGGGAAGCGCCACACGTT-3') designed from TM1–TM2 of the Na⁺,Cl[−]-dependent transporter family was synthesized and 5' end-labeled with ³²P. After screening a bovine retina λ ZAP cDNA library, the inserts were excised from the positive phages with R408 helper phage and sequenced by the dideoxy chain termination method as described [20–22]. Both strands were sequenced.

2.2. Northern blot analysis

Poly(A)⁺ RNA (10 μ g) was extracted from retina, cerebral cortex, cerebellum and liver of bovine using the Fast Track mRNA Isolation kit (Invitrogen). The cDNA region specific for bv7-3s (corresponding to nucleotides 776–1193) and bv7-3 (1315–3229) and the common region of both cDNAs (corresponding to nucleotides 1–378 of bv7-3s) were labeled with ³²P by random priming (10⁵ cpm/ml). Northern blot analysis using each cDNA probe was performed as described [20].

2.3. Immunofluorescence analysis of HEK 293T cells transfected with myc-tagged bv7-3 and myc-tagged bv7-3s

bv7-3 or bv7-3s was subcloned in the mammalian expression vector pCAGGS [23]. The sequence of the c-myc epitope tag EQKLI-SEEDLN was inserted just before the stop (TAG) codon in bv7-3 cDNA and in bv7-3s cDNA by PCR amplification. The PCR product was digested with *NheI*-*EcoRI* for bv7-3 and *EcoRI*-*EcoRI* for bv7-3s, and ligated into similarly digested pCAGGS-bv7-3 and pCAGGS-bv7-3s respectively.

For transient transfections, HEK 293T cells were grown on chamber slides (LAB-TEK) in Dulbecco's minimum essential medium (DMEM; Nikken Bio Medical Laboratory) supplemented with 10% fetal bovine serum at 37°C in a CO₂ incubator. The cells were transfected by Lipofectamine (Gibco BRL) with 0.25 μ g of the expression plasmid myc-tagged bv7-3/pCAGGS or myc-tagged bv7-3s/pCAGGS and left for 3 days in the CO₂ incubator.

For immunofluorescence experiments, the transfected cells were fixed with 4% paraformaldehyde and 0.21% picric acid in 0.1 M phosphate-buffered saline (PBS) for 1 h. These cells were stained by immunocytochemistry using anti-myc 9E10 antibodies (ATCC; 1:3) and anti-mouse IgG (Jackson; 1:500) as described in [24]. Immunofluorescence was visualized with a photomicroscope Optiphot (Nikon).

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3. Results

3.1. Cloning

We isolated 14 positive clones from a bovine retina cDNA library, using a 62-mer oligonucleotide probe originated from the Na⁺,Cl⁻-dependent transporter gene family. Sequence analysis revealed that seven independent clones shared identi-

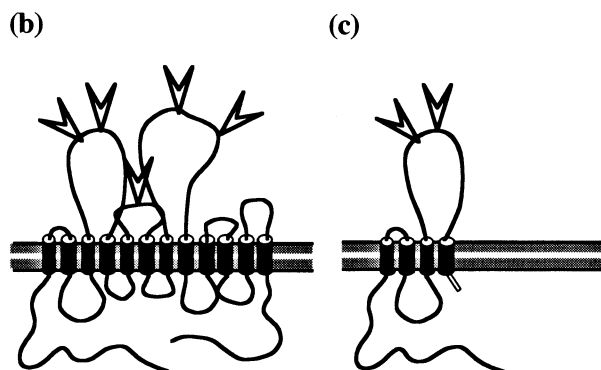
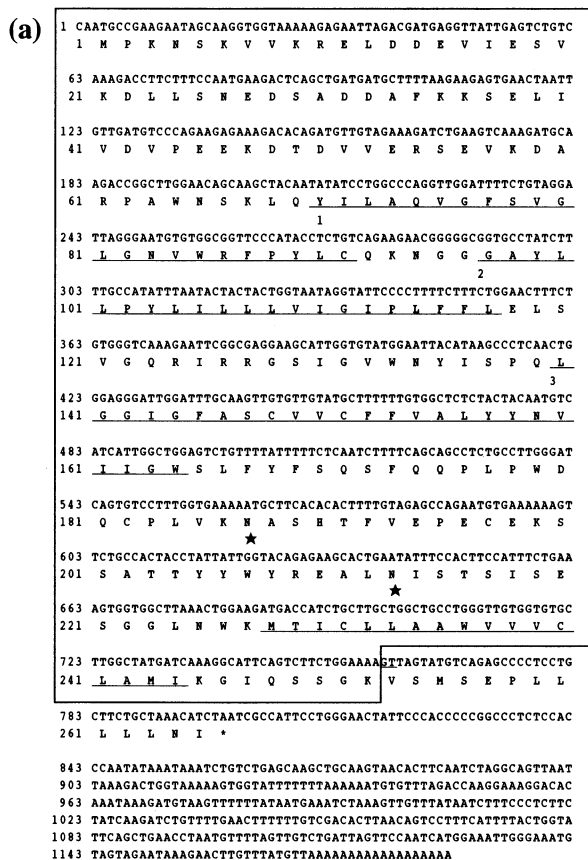


Fig. 1. Nucleotide and deduced amino acid sequences of bv7-3s cDNA (a), and schematic representation of bv7-3 (b) and bv7-3s (c) showing proposed orientation in the plasma membrane. a: The coding region is numbered starting from the putative translation initiation codon. Putative transmembrane regions are marked by solid bars. N-Glycosylation sites are marked with asterisks. The region common to bv7-3 is boxed. b,c: bv7-3 and bv7-3s share 252 N-terminal amino acids as represented by the darkened line. bv7-3 consists of 12 putative transmembrane domains, like other Na⁺,Cl⁻-dependent neurotransmitter transporters, while bv7-3s contains only four such domains.

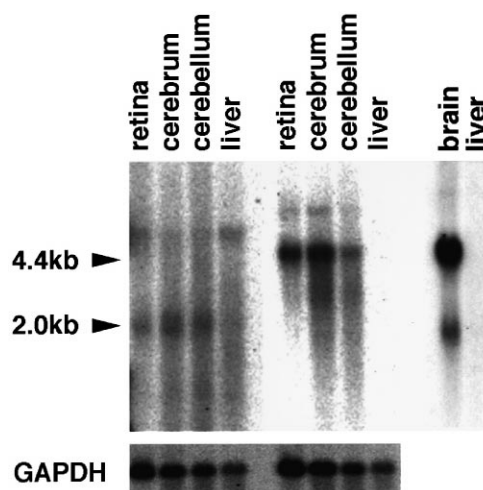


Fig. 2. Northern blot analysis using probes specific for bv7-3s (left) and bv7-3 (middle) and common to both (right). Expression of mRNAs for bv7-3s and bv7-3 was detected in retina, cerebrum, and cerebellum, but not in liver with a size of 2.0 kb and 4.4 kb respectively (left and middle). Control hybridization of each blot with the GAPDH probe is shown underneath. mRNA for bv7-3s was relatively faint compared to that of bv7-3 (right).

cal nucleotide sequences. These seven clones were divided into two groups according to their length: those with 12 transmembrane domains (bv7-3) and those with only four transmembrane domains (bv7-3s, bv7-3s2, bv7-3s3). The nucleotide sequence data of bv7-3, bv7-3s, bv7-3s2 and bv7-3s3 have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB02854, AB02855, AB02856 and AB02857, respectively.

bv7-3 displayed 90.4% amino acid identity to rat orphan transporter v7-3-2 which was cloned from a rat midbrain cDNA library and belongs to the Na⁺,Cl⁻-dependent transporter family [20]. The initiating methionine was proposed in comparison with another orphan transporter NTT4/rXT1 [25,26], since the sequence of the 5' seven amino acids (MPKNSKV) was the same as that in NTT4. bv7-3 was 3217 bp long with an open reading frame of 2187 bp which could encode a protein of 729 amino acids with a calculated molecular mass of 82 kDa. The Kyte-Doolittle hydrophobicity profile [27] suggested the presence of 12 stretches of hydrophobic residues that represent potential transmembrane domains as in other transporters. Potential sites for Asn-linked glycosylation were located in the second, third and fourth extracellular loops, which were different from other orphan transporters including v7-3-2 (Fig. 1a,b).

The shorter cDNAs, bv7-3s, bv7-3s2 and bv7-3s3, are quite similar, although not identical since they had different-length 5' and 3' non-coding regions. They encoded an identical protein containing 265 amino acid residues. The N-terminal 252 amino acids of bv7-3s were the same as those of bv7-3. The short variants contained only four putative transmembrane domains, while bv7-3 consists of 12 putative transmembrane domains (Fig. 1b,c). We selected bv7-3s for further analyses as a short variant of the transporter among bv7-3s, bv7-3s2 and bv7-3s3.

3.2. Northern blot analysis

To determine whether the short variants actually exist in

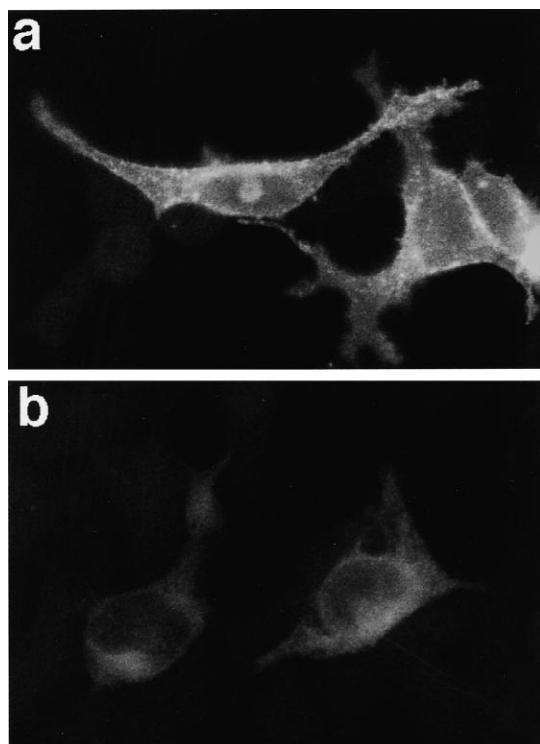


Fig. 3. Immunofluorescence analysis of the cellular distribution of myc-tag bv7-3 (a) and myc-tag bv7-3s (b) in transiently transfected HEK293T cells. Myc-tagged bv7-3 was found at the cell surface, whereas myc-tagged bv7-3s was detected in the intracellular membrane. Magnification, $\times 180$.

vivo, we investigated mRNA expression of bv7-3 and bv7-3s by Northern blot analysis. Using cDNA probes specific for bv7-3, we detected bands in retina, cerebral cortex, and cerebellum, but not in liver, with a size of 4.4 kb. With the use of a cDNA probe specific for bv7-3s, transcripts of bv7-3s were detected in the same tissues as bv7-3 with a size of 2.0 kb (Fig. 2). Then, for quantitative estimation, we performed Northern blot analysis using a cDNA probe common to bv7-3s and bv7-3, and found that the detected band for bv7-3s was relatively faint compared with that of bv7-3.

3.3. Immunofluorescence analysis

The substrates of bv7-3 have yet to be determined. The proteins of the members of the Na^+ , Cl^- -dependent transporter family are located in the plasma membrane where they transport substrate from the extracellular into the intracellular space. To confirm that bv7-3 is expressed at the cell surface to take up the substrates, and to investigate the role of the short variant bv7-3s, we determined the subcellular localization of their protein in HEK 293T cells transiently transfected with myc-tagged bv7-3s and myc-tagged bv7-3 by immunofluorescence microscopy. bv7-3 was found on the plasma membrane in the myc-tagged bv7-3-transfected cells. Interestingly, myc-tagged bv7-3s showed a pattern of intracellular membrane staining (Fig. 3).

4. Discussion

We cloned a novel type of splicing variant of transporter (bv7-3s) with only four putative transmembrane domains. We

determined that these mRNAs are spliced from a single gene by Southern blot analysis (data not shown). bv7-3s has the nucleotides GT [28], the 5' consensus splice site for splicing just after the common region with bv7-3, although the sequence around AAA/GTTAGT was not completely the same as the consensus donor sequence (C/A)AG/GURAGU (the intron-exon boundary is denoted by the vertical line) (Fig. 1b) [29]. This result indicates that bv7-3s arises when the pre-RNA for this transporter gene is not spliced but cleaved and polyadenylated at internal poly(A) addition sites in the same way as reported for the secreted immunoglobulin μ heavy chain (Ig μ) [30,31]. Otherwise, bv7-3s may be produced via splicing with exons different from those of bv7-3 and using the associated internal polyadenylation sites, as reported for calcitonin/CGRP [32]. The genomic sequence will unveil details of the construction of exons.

The result that the mRNA expression of bv7-3s was relatively faint compared with that of bv7-3 was also confirmed by reverse transcription PCR (data not shown). This could be due to (1) splicing regulation; splicing for the short variants occurs at a low frequency, or it is amenable to regulation by environmental signals, or (2) instability of the short variants with different 3' non-coding regions.

Immunofluorescence analysis revealed that myc-tagged bv7-3s localized in the intracellular membrane whereas myc-tagged bv7-3 was detected mainly in the cell surface in HEK 293T cells. Turner et al. demonstrated the COOH-terminal is important for cellular sorting of the sodium-dependent glucose cotransporter [33]. Perego et al. showed that on deletion of the last 49 COOH-terminal amino acids, the betaine transporter failed to reach the basolateral cell surface resulting in loss of transport activity at the cell surface [34]. Olivares also showed that deletion of more than 34 amino acids of the carboxy-terminus of GlyT1 produced a progressive decrease in transport activity and that those carriers were no longer found in the membrane [24]. These findings suggest that the carboxy-terminus of bv7-3 could be involved in the membrane insertion process and that bv7-3s, by deletion of almost half the carboxy-terminus of bv7-3, could no longer reach the plasma membrane. The protein encoded by bv7-3s seems not to have transporter activity at the cell surface but to be a by-product of posttranscriptional regulation of bv7-3 or to have certain functions at the intracellular membrane.

Whether the splicing of the short variant affects the transport activity remains unknown since we have not identified the substrate of bv7-3 [20,25,26,35,36]. In previous reports however, short transporter variants were detected by Northern blot analysis [10,35–37] suggesting that short splicing variants are common and affect the regulation of transporter activity at the posttranscriptional level. And the posttranscriptional regulation may be important for the stage of disease, as indicated in a recent report that sporadic amyotrophic lateral sclerosis patients have multiple abnormal EAAT2 variants caused by intron retention and exon skipping which result in loss of EAAT2 protein activity [38].

In conclusion, in the present study, we cloned novel splicing variants of transporter, with the following traits. (1) They share an N-terminal region with four putative transmembrane domains and the shorter bv7-3s ends with 13 different amino acids and a non-coding 3' region. (2) Their mRNAs are present in the same tissue. (3) bv7-3s is localized in the intracellular membrane, bv7-3 mainly at the cell surface. The func-

tional role of this short-form transporter remains to be elucidated. The results of our study are expected to offer insights into the molecular mechanism of splicing of transporters.

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