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Short sequence-paper

A liver-specific isoform of the betaine/GABA transporter in the rat: cDNA sequence and organ distribution [1]

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Abstract

We report the cloning of a 2.2 kb cDNA encoding a Na^+- and Cl^--dependent betaine/GABA (γ-aminobutyric acid) transporter from rat liver poly(A^+) RNA. 5′-RACE revealed an additional 355 bases 5′ to the 2.2 kb cDNA sequence. Northern analysis demonstrated two (2.2 kb and 2.6 kb) mRNA isoforms in rat liver. Betaine transporter mRNA was also detected in the brain, spleen, lung, and kidney using the 2.2 kb cDNA clone as a probe. Only the 2.6 kb mRNA from the liver hybridized with the 5′-RACE product.

Keywords: Betaine; Osmolyte; Membrane transporter

Betaine (*N*-trimethylglycine) is a protective intracellular osmolyte that is accumulated by cells in response to hypertonic stress [1], and is associated with the kidney's ability to form a concentrated urine [2]. Betaine also acts as a methyl donor in the detoxification of homocysteine [3] and may therefore be important in the etiology of atherosclerosis [4]. It is produced in mammalian liver from choline by the sequential action of choline oxidase and betaine aldehyde dehydrogenase [5].

In the dog, betaine is accumulated (intracellularly) by a sodium and chloride-dependent betaine/GABA transporter (BGT1), which is expressed at highest levels in the kidney [6]. Three isoforms (designated A, B, and C) of the canine mRNA have been reported which are distinguished by transcription start sites at three different 5′-end exons [7]. Takenaka et al. [7] found all three isoforms in the kidney, two occurred in the brain, and only one was present in the canine liver. None of the three canine isoforms differed in amino acid coding regions. In the present study, we report the identification, cloning, and expression in *Xenopus* oocytes of two rat liver betaine/GABA transporters, one of which is unique to the liver, and contains an extended open reading frame.

We designed two oligonucleotide primers for PCR based on conserved sequences among the sodium- and chloride-dependent osmolyte/neurotransmitter transporter gene family (SNF). The sense primer was 5′-CTGGGCAATG-TITGGAGGTTCC-3′ and the antisense primer was 5′-CATGAAGCCCACGATGGAGAAGA-3′. These were used to amplify Sprague–Dawley rat liver poly(A)-selected RNA by RT-PCR using M-MLV reverse transcriptase and Taq DNA polymerase (Gibco/BRL). Agarose gel electrophoresis was then used to visualize the resulting products. A band of the expected molecular weight was excised from the gel, treated with β-agarase (New England Biolabs), phenol/$CHCl_3$-extracted, ethanol precipitated, blunt-ended with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase and ligated into dephosphorylated *Sma*I-cut Bluescript. *Escherichia coli* were transformed by electroporation and selected on ampicillin/X-gal plates. Ten clones were selected for sequence analysis. One clone contained an 866 bp PCR product which showed 85% identity with the nucleotide sequence of the dog kidney betaine transporter. A rat liver cDNA library, prepared using SuperScript® reverse transcriptase (Gibco/BRL) in pSport 1, was screened using the cloned RT-PCR product as a probe. A 2.2 kb cDNA clone was isolated and sequenced (Fig. 1).

An organ blot of poly(A^+) RNA from rat (Clontech) was probed with ^{32}P-labelled 2.2 kb cDNA (Fig. 2). Unlike the dog, rat liver had higher levels of betaine

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[1] The nucleotide sequence has been assigned GenBank accession number U28927.

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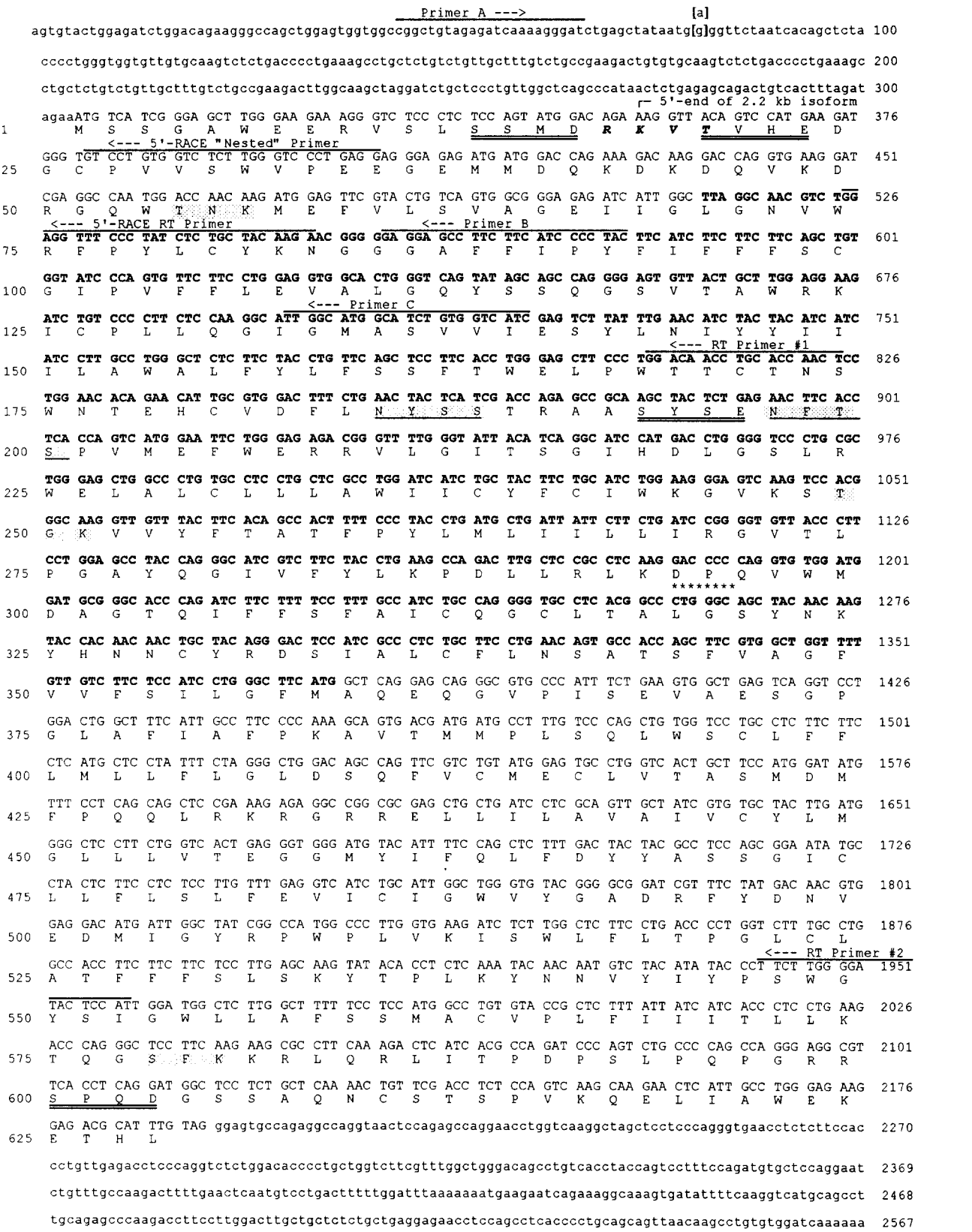

Fig. 1. Rat liver betaine/GABA transporter sequence. Sequences from the 2.2 kb cDNA clone and the 5′-RACE product are combined to give the putative 2.6 kb sequence. The 5′-end of the 2.2 kb cDNA clone is indicated. Primers discussed in the text are indicated by overlined text. Double underlined text indicates casein kinase II phosphorylation site consensus sequences. Shaded text indicates protein kinase C phosphorylation site consensus sequences. Text which is both shaded and underlined indicates potential glycosylation sites. Bold italicized text indicates a protein kinase A phosphorylation site consensus sequence (amino acids 17–20). The PCR product used to screen the cDNA library is indicated by boldface nucleotides. The Genbank accession number of this sequence is: U28927.

transporter mRNA than kidney, or indeed, than any other of the tested organs. The diffuse labelling of mRNA bands in the kidney lane indicates two or more isoforms are present which are poorly resolved. In the liver, two intense bands are well-resolved (cf. Fig. 4), one which is approx. 2.2 kb, and another which is approx. 2.6 kb. The kidney isoforms are all smaller than the large liver isoform. Brain, spleen, and lung express a single isoform of betaine transporter mRNA, whereas heart, skeletal muscle, and testis do not. The brain appears to express only a 2.2 kb isoform of a size similar to the smaller liver isoform. The spleen and lung both express isoforms intermediate between the larger (2.6 kb) and smaller (2.2 kb) liver isoforms. Expression of a betaine transporter in lung and spleen has not been previously reported, and it is not clear what function it may have in these organs. There is no evidence in the rat brain of the predominant 5 kb isoform observed in mouse brain [8].

5′-RACE [9] was performed on rat liver poly(A^+) RNA using the 5′-AmpliFINDER® RACE kit (Clontech). The two 5′-RACE primers indicated in Fig. 1 were used, as well as the kit's 5′-anchor oligonucleotide. Agarose gel electrophoresis of the PCR reaction product revealed that a single ≈ 450 bp product was amplified (results not shown). The band was excised from the gel, ligated into pSport 2, and used to transform *E. coli*. Seven independent clones were isolated and sequenced. The sequences were all identical except for a single base (number 81 in Fig. 1) which was an 'A' in 4 clones and a 'G' in 3 clones (Fig. 1). This may reflect an error in Taq polymerase transcription, but more likely reflects heterogeneity in the 5′-non-coding region of the mRNA. Note that the sequence obtained matched the 5′-most 25 bases from the 2.2 kb cDNA (Fig. 1, nucleotide #356) and had a high degree of homology to mouse and dog betaine transporter sequences for a further nine bases.

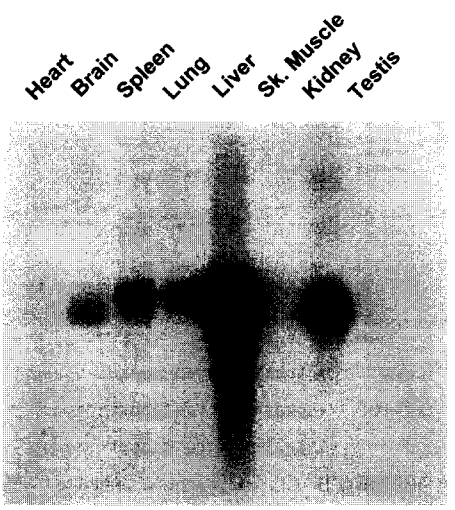

Fig. 2. PhosphorImager™ (Molecular Dynamics) radiogram of a Northern blot probed with the 2.2 kb cDNA, showing (from left) poly(A^+) RNA (2 μg per lane) from rat heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis.

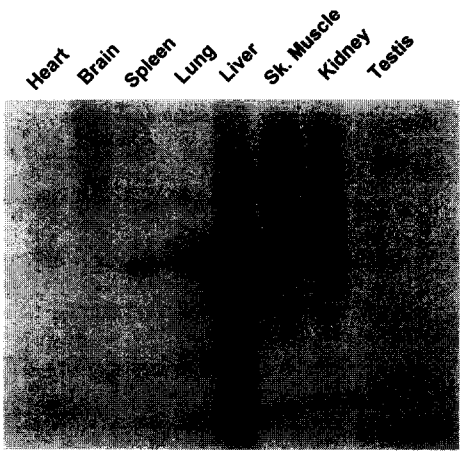

Fig. 3. PhosphorImager™ radiogram of a Northern blot probed with 5′-RACE product, showing (from left) poly(A^+) RNA (2 μg per lane) from rat heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis.

The 5′-RACE product (directly excised and purified from the agarose gel) was ^{32}P-labelled and used to probe the same Northern blot used in the experiment shown in Fig. 2. Fig. 3 shows that in contrast to Fig. 2, only the liver is labelled by the 5′-RACE product, indicating that the larger isoform (cf. Fig. 4) is specific to the liver. This is in contrast to the dog, which has only one isoform (type B) in the liver; and furthermore, this isoform is not unique to the liver, but is also found in the kidney and brain as well [7].

Because the family of NaCl-osmolyte/neurotransmitter cotransporters (SNF) is so large, and since both the tissue distribution and the relative quantities of rat Na^+, Cl^-, betaine-cotransporter isoforms were at such variance with what had been reported in either dog [6,7] or mouse [8], it seemed prudent to insure that the 5′-RACE product was truly a part of the betaine/GABA transporter sequence, and not a serendipitous homologue. In order to verify this, four different RT-PCR reactions were prepared which would amplify across the junction between the 2.2 kb cDNA and the 5′-RACE product. If the different series of primers (based on the two known sequences) were able to amplify across the apparent RACE/cDNA junction to give PCR products of the expected size (thereby excluding the possibility of inclusion of one or more introns), and also to produce sequences that hybridize specifically with the expected mRNA on a Northern blot, the possibility of serendipitous homology having produced an artefactual 5′-RACE product becomes remote.

Two reverse transcriptions were performed on rat liver poly(A^+) RNA using either 'RT Primer #1' or 'RT Primer #2' as indicated in Fig. 1. After RNA hydrolysis and removal of excess RT primers, each cDNA preparation was split into two parts, and used as substrate for PCR using the indicated primer pairs A and B or A and C (Fig. 1). In all four cases, agarose gel electrophoresis revealed

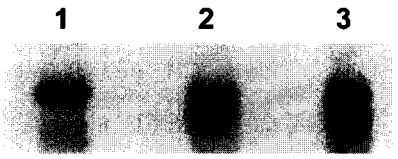

Fig. 4. PhosphorImager™ radiogram of a Northern blot of rat liver poly(A$^+$) RNA, 4 μg/lane. Three lanes were run in parallel on a formaldehyde/agarose gel, blotted onto a nylon membrane, UV-cross-linked and air-dried. The three lanes on the blot were cut to separate them, using notches for future alignment. Lane 1 was probed with the 5′-RACE product (as in Fig. 3), labelled with [α-^{32}P]dCTP. Lane 2 was probed with an RT-PCR product prepared using cDNA reverse transcribed from rat liver poly(A$^+$) RNA using as a primer, an oligonucleotide which was the complement of the sequence designated 'RT Primer #1' in Fig. 1 (i.e., 5′-AGTTGGTGCAGGTTGTCCA-3′); and the primer pair 'A' and 'B' (i.e., primer 'A' was 5′-CGGCTGTAGA-GATCAAAAGGGAT-3′, and primer 'B' was 5′-GTAGGG-GATGAAGAAGGCTCCTC-3′) was used for PCR amplification. Lane 3 was probed using an RT-PCR product prepared using RT Primer #1, and the primer pair 'A' and 'C'. After probing, the strips were re-aligned and exposed to the PhosphorImager™ storage screen. Because of differences in background and intensity of labelling, exposures were optimized for each lane. The 5′-RACE product hybridizes only with the 2.6 kb mRNA, but primer pairs selected from within the 5′-RACE product and the 2.2 kb cDNA clone produce a PCR product which hybridizes with both mRNA isoforms.

that single bands of the expected molecular weights were amplified. Primer B, which is conserved in the mouse cDNA, is broken by an intron in the mouse gene (Burnham, C.E., unpublished results) which if conserved in the rat, would have produced amplification products larger than expected in the A-C amplification had genomic DNA been amplified. The A-B and A-C amplimers from each reverse transcription reaction were identical in molecular weight, but the amplified products from RT Primer #1 were more abundant, based on intensity of ethidium bromide staining. This was to be expected since the first strand cDNA produced using RT primer #2 needed to be significantly longer before the amplifiable regions were reached. The products from A-B/RT Primer #1 and A-C/RT Primer #1 were excised from an agarose gel and purified using Qiaex® spin columns (Qiagen). The two purified PCR products (along with the 5′-RACE product) were then ^{32}P-labelled, and each one was then used to probe one of three lanes from a rat liver poly(A$^+$) Northern blot (Fig. 4). As shown in Figs. 3 and 4, the 5′-RACE product labelled only the larger isoform from the liver mRNA. Both of the two trans-junctional amplimers labelled both liver isoforms, confirming that the RACE product was indeed a part of the rat liver betaine/GABA transporter mRNA large isoform.

The combined putative 2.6 kb transcript (shown in Fig. 1) has an open reading frame encoding 628 amino acids. The first 14 amino acids are not encoded by the canine [6,7], mouse [8], or human [10] betaine/GABA transporter cDNA's. It is not known whether these amino acids are actually translated. Percent homologies with the other species are: mouse, 96%; dog, 88%; and human, 86%. A consensus sequence [11] for casein kinase 2 phosphorylation (SSMD, Fig. 1) occurs in the novel coding region, and does not occur in other isoforms. Other sites of possible phosphorylation and glycosylation were determined by a scan of the PROSITE data bank [11] and are indicated in Fig. 1.

The 5′-RACE product was ligated onto the 2.2 kb cDNA 5′-end and cloned. The ligation and orientation were confirmed by restriction analysis. RNA transcribed from each of the two isoforms was then injected into *Xenopus* oocytes, and [^{14}C]betaine (synthesized from [^{14}C]choline [12]) and [^{3}H]GABA uptake was measured. Uptake of [^{3}H]GABA was completely dependent on the presence of Na^+ and Cl^-. Uptake of 50 μM [^{14}C]betaine was inhibited by betaine (5 mM), L-alanine (5 mM), and GABA (5 mM); but not by methylaminoisobutyric acid (5 mM), proline (5 mM), glutamine (5 mM), glycine (5 mM), or taurine (5 mM). Uptake into oocytes were measured using at least two frogs, and at least 5 oocytes per point per frog. K_M values for GABA and betaine were 0.3 and 0.4 ($\pm$0.1) mM, respectively, for both isoforms (results not shown). It is more appropriate to refer to this transporter as a 'betaine' transporter than a GABA transporter although its affinity for GABA is greater than for betaine. There would be little hepatic GABA for the betaine/GABA transporter to carry because of the presence in the rat liver of another GABA transporter, GAT-2, which is expressed at high levels in the liver and has a 10-fold higher affinity for GABA [13].

The fact that both betaine transporter isoforms function similarly is consistent with the work of Mabjeesh and Kanner [14], who found that the amino and carboxyl termini of a closely related GABA transporter from rat brain are not required for its function. Indeed, a computer analysis of other betaine transporter sequences using MACAW [15], indicates a reduced homology among the betaine transporter sequences prior to the first methionine in the 2.2 kb cDNA coding region.

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