Identification and tissue distribution of two differentially spliced variants of the rat carnitine transporter OCTN2

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Abstract In this paper we show that the only known Na^+ dependent transporter of carnitine in mammals, organic cation transporter number 2 (OCTN2), is subject to differential splicing. Cloning of OCTN2 in different rat tissues identified two splicing variants. We have developed a real time quantitative polymerase chain reaction method for quantification of these splice variants. Both splice variants could be detected in all tissues examined with a relative abundance of 0.1–1% of the full length transcript. We also draw attention to the previously described mutations in clinical examples of primary carnitine deficiency in humans where the described mutations appear to be those of a splicing or mis-splicing event.

Key words: Systemic carnitine deficiency; Real time quantitative PCR (TaqMan); Splice variants; Organic cation transporter; Carnitine

1. Introduction

Novel organic cation transporter number two (OCTN2), is the only known sodium co-transporter of carnitine, an essential component of long chain fatty acid transport into the mitochondrial matrix for oxidation. As omnivores, we rely on a dietary intake of approximately 70% of our daily requirements for carnitine. Uptake, biosynthesis and renal reabsorption are all vital components of a not yet well understood balance of carnitine in our bodies, where the distribution of plasma carnitine into tissues plays an important role. Tissue concentrations of carnitine vary over a wide range with the highest local concentrations in humans found in skeletal muscle and heart (3–5 mM) [1]. However in other animal tissues, concentrations of carnitine have been described as high as 2000 fold that of plasma [2], reaching concentrations of up to 50 mM in the caudal epididymis of rats. This mosaic of tissue carnitine stores illustrates the important role played by active carnitine transporter protein(s) in mammals.

The kinetics of carnitine transport differ from tissue to tissue and are even variable within the one tissue type showing both high and low affinities for carnitine (muscle [3,4]; kidney [5]; and liver [6,7]). Over-expression of the OCTN2 protein in cultured cells has demonstrated that this transporter has a high affinity for carnitine, and its kinetics of transport are consistent with some of those found in whole tissues [8–10]. In fact, patients with systemic carnitine deficiency (SCD), an hereditary disease associated with myopathy, muscle and metabolic disorders, have now been shown definitively to carry mutations in their OCTN2 genes.

The mutations described so far for these patients have been mostly point mutations [10–19], with only one publication [20] describing mutations at the level of transcription; a deletion and a partial intronic insertion. In a further report from Burwinkel et al. [21] a nonsense mutation in exon V is described as being the cause of the observed carnitine deficiency, however it was noted that in the two unrelated patients there was an associated splicing abnormality at the intron VI/exon VII boundary. The novel transcript was identified by reverse transcription-polymerase chain reaction (RT-PCR) but despite extensive sequencing, the researchers could find no causal alteration of the genomic DNA in the vicinity of the splice boundary, compatible with abnormal splicing.

The rat OCTN2 gene shares 87% sequence homology with the human cDNA sequence, and 85% amino acid similarity [22], its kinetics of transport mimic those of the Na^+ dependent transporter in humans except for a slight substrate impartiality between carnitine and the more general cation TEA [8]. In the process of cloning the rat OCTN2 gene for further study we observed a number of different mRNA transcripts indicating possible differential splicing of this gene in rats. We analysed these transcripts using quantitative real time PCR in different rat tissues and discuss our findings in relation to the mutations reported for this gene in humans.

2. Materials and methods

2.1. Preparation of RNA/initial cloning

Three healthy male Sprague–Dawley rats were killed by decapitation. Liver, heart, kidney, testis, small intestine and skeletal muscle from the hind limb were excised and immediately frozen in liquid nitrogen until further use. Total RNA was isolated from 30 mg frozen tissue using Qiagen mini-prep columns (Qiagen AG, Basel, Switzerland) according to the manufacturer's instructions. An additional proteinase K treatment (Promega, Madison, WI, USA) of 6 U (10 min at 55°C) was needed prior to isolation of RNA from skeletal muscle and heart.

cDNA was made by reverse transcribing 5 μg total RNA using the enzyme SuperscriptII (Life Technologies AG, Basel, Switzerland) and random hexamer primers (Microsynth, Switzerland) according to the manufacturer's directions. DNase treatment was deemed unnecessary for quantitative PCR as the amplicons for GAPDH and OCTN2 span intron/exon boundaries and genomic DNA contaminants would not amplify under the limiting thermocycling conditions used. Initial cloning of rat OCTN2 was of a PCR product amplified with forward primer 5′-GGCATGCGGGACTACGACGA and degenerate reverse

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primer 5′-RGYGKCCCTRGAGGAAGGTG cloned into the vector pCR-2.1 (Invitrogen, The Netherlands).

Sequencing of OCTN2 clones was done by Microsynth (Switzerland), single strand sequencing in four overlapping segments using two vector and two internal sequencing primers.

2.2. OCTN2 mRNA quantitation

Real time quantitative PCR (TaqMan, Applied biosystems, Rotkreuz, Switzerland) was performed on an ABI PRISM 7700 sequence detector to determine the relative expression levels of the various splice variants observed in rat tissues (see Fig. 1).

Primer pairs were chosen with a constant forward primer, a FAM/TAMRA modified oligo as the reporter probe, and a variable reverse primer which, by design, was the crucial determining factor in amplifying the different transcripts. This primer was placed across the exon/exon boundary amplifying only that transcript which corresponded to one of those we had observed through our sequencing results.

The forward primer 5′-TGGGAGTACAACAAGGACGACGTCTT-3′ (bp 348–371, where 1 is the start codon ATG), non-coding strand probe 5′-TCCCGAAATGAAGGAGCCCATCA-3′ (bp 483–461), primer B-full length transcript 5′-ACATTCTTGCGACCAAACCTG-3′ (bp 515–495), primer B-exon III deletion 5′-TTGAAAGAATTTCTGTTCCTG-3′ (bp 671–652+497–495) and primer B-88del 5′-GCAGCATCCTCCAGTCTCTGTC-3′ (bp 778–761+497–493) were used.

For each reverse primer, the final 3–5 nucleotides (3′-end) corresponded always to the last portion of exon II. Internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used previously published primers and probe [23]. cDNA corresponding to 25 ng of reverse transcribed total RNA was amplified in a 25 μl volume reaction using TaqMan universal PCR Mastermix (PE biosystems) in triplicate assays for both rOCTN2 targets and the endogenous control GAPDH. Primers (Microsynth, Switzerland) were used at a concentration of 300 nM each, FAM/TAMRA fluorophore/quencher reporter probe (Eurogentec, Belgium) at a concentration of 100 nM. Thermocycling conditions were as for standard TaqMan protocol (PE biosystems), 10 min denaturation at 95°C followed by 40 cycles of 95°C for 15 s, 60°C for 60 s.

The primers and probe were validated for amplification efficiency by running the reaction at five different dilutions of template and subtracting the results obtained for the control GAPDH from those of the target, this established that the amplification efficiency was the same for all primer pairs. Thereafter, analysis of the results obtained was based on the Ct method of calculation, where Ct is the cycle number at which the fluorescence of the sample crosses a given threshold. This threshold is set within the linear log phase of each reaction, i.e. where amplification of the product is still exponential and therefore a direct correlation can be made with the relative amounts of starting template in each reaction.

For more information see the web site of Applied Biosystems at the following address http://www.appliedbiosystems.com/molecularbiology/about/pcr/sds/5700_sds/5700app.html#section2.

The expression levels of the full length carnitine transporter in the liver was arbitrarily taken as the 'calibrator' for all calculations and all other tissues were normalised accordingly. Results are given as expression of each transcript relative to the full length in liver ± S.D. for each tissue of three animals.

2.3. Computer analysis

Prediction of protein tertiary structure was performed with the online-server HMMTOP prediction of transmembrane helices and topology of proteins version 1.1 [24] and Prosite databases (SIB, Geneva, Switzerland version 99.07) [25].

3. Results/discussion

The rat carnitine transporter OCTN2 was amplified by PCR from liver, kidney and muscle tissues as described in Section 2 and cloned into PCR TOPO cloning vector pCR-2.1. Screening of the resultant clones showed the presence in all tissues of shorter than expected inserts (roughly one in 10 clones screened). Sequencing of the clones revealed three distinct forms of cDNA. Aside from the full length published rat OCTN2 a second transcript was obtained from the clones derived from liver RNA which we termed '88del' and a third termed 'exon III' from both muscle and kidney clones (see Fig. 2).

Comparison with the human OCTN2 genomic sequence from the database [26] (the rat genomic not being available to date but sharing a 87% cDNA sequence homology) revealed exon III to be a product of splicing where, as its name suggests, the whole of exon III was spliced out resulting in a predicted truncated protein of 226 amino acids. The second, termed 88del for what would be a deletion of 88 amino acids in any translated product, was missing exon III and a part of exon IV, the sequence resuming in the middle of exon IV at a cryptic splice site. At this point in the cDNA the rat sequence varies from that of the human genomic sequence, a **c** to **a** base pair difference changing a **ccg** motif into a **cag** potential splicing signal. It is at this dimorphism in a normal rat that the resumption of the transcript partway through exon IV is resumed encoding a predicted protein of 469 amino acids (see Fig. 3). In a normal transcript, where all 10 exons are included, this cryptic splice site in the middle of exon IV would go unnoticed. It is only when exon IV is spliced out that it is presented as a false intron acceptor site and coding resumes partway through the exon.

A strategy was derived whereby we could check the abundance of these splice variants in relation to the full length sequence and whether or not the variants were tissue specific. Previously published studies on the mRNA expression of OCTN2 have consisted solely of Northern blot analysis using a probe randomly primed off the entire or a large (>900 bp) part of the mRNA and therefore retain inherent problems of questionable specificity given the large degree of sequence ho-

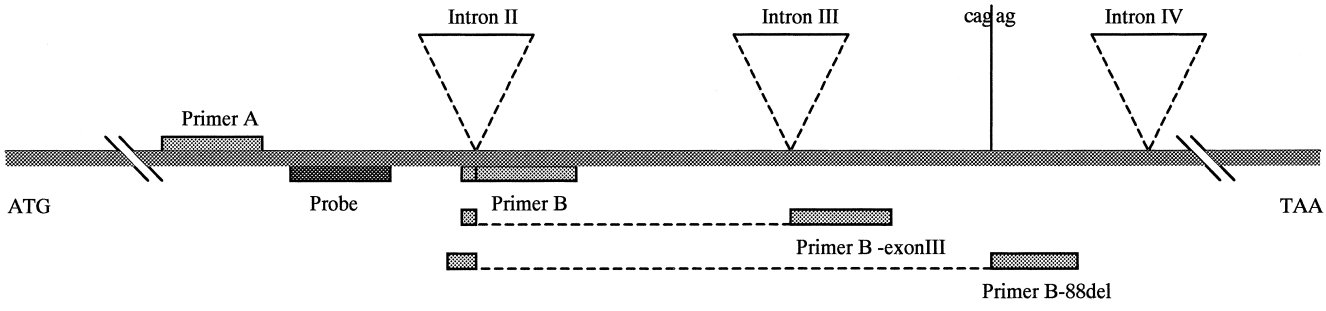

Fig. 1. Schematic illustration of the strategy employed for quantitative PCR of the different mRNA transcripts from the rat OCTN2 gene depicting the location of forward and reverse primers and the position of the modified oligonucleotide reporter probe.

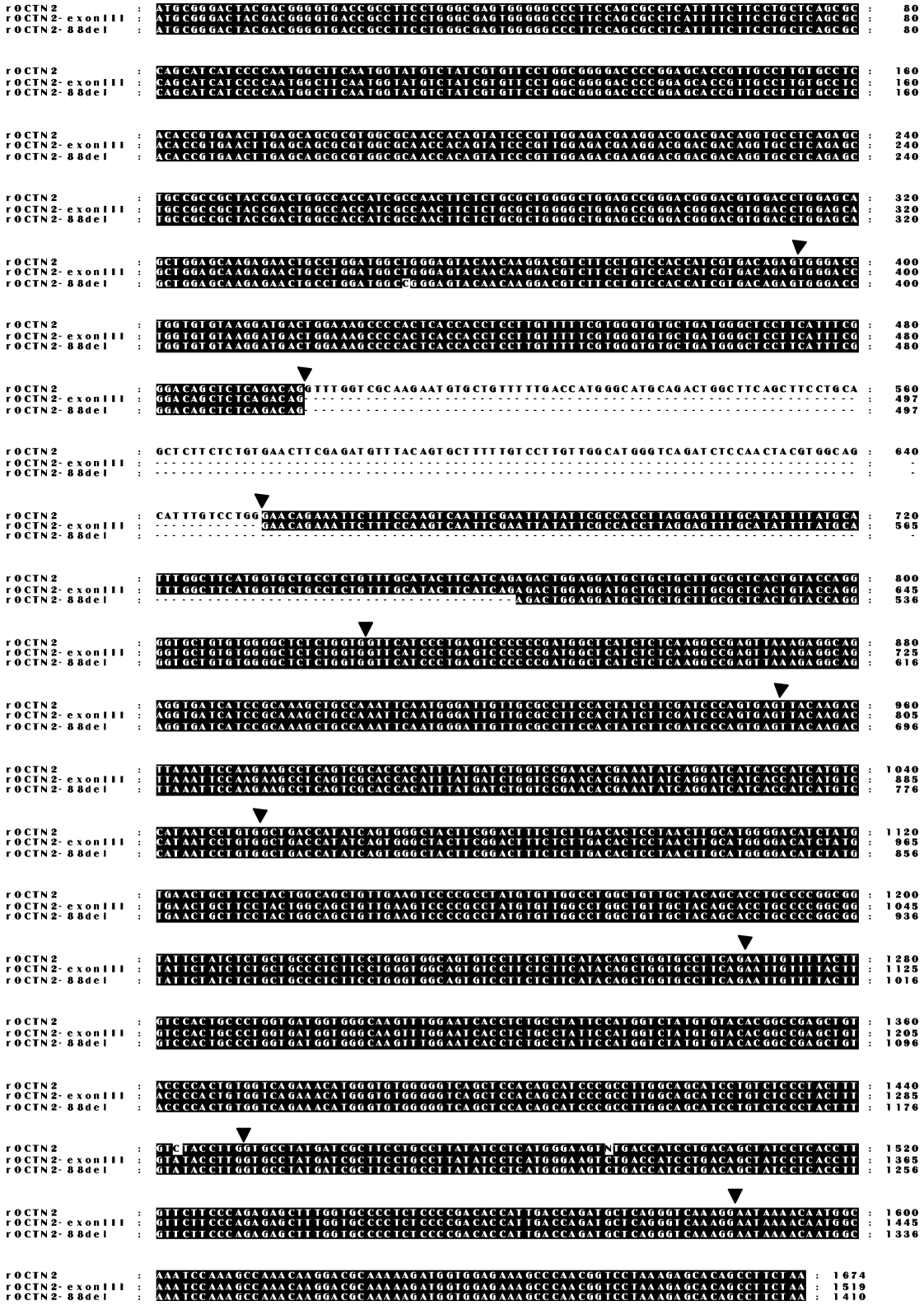

Fig. 2. cDNA alignment of sequences obtained for the full length rat OCTN2 and the two splice variants. Arrows signify positions of introns as derived from the human genomic sequence. The precise boundary between intron and exon was taken always with a *cag* or *tag* consensus donor site as the last 3 bp of the intron, thus defining the first base of the following exon.

mology shared by the known members of this expanding family of transporters. Doublet bands have been previously noted in Northern blot analysis of both human and rat OCTN2 [26,22,32] but their true identity remained uncharacterised. To avoid these problems of high family homology we chose the method of quantitative PCR and developed a strategy whereby we could specifically target each chosen mRNA species for individual amplification and analysis.

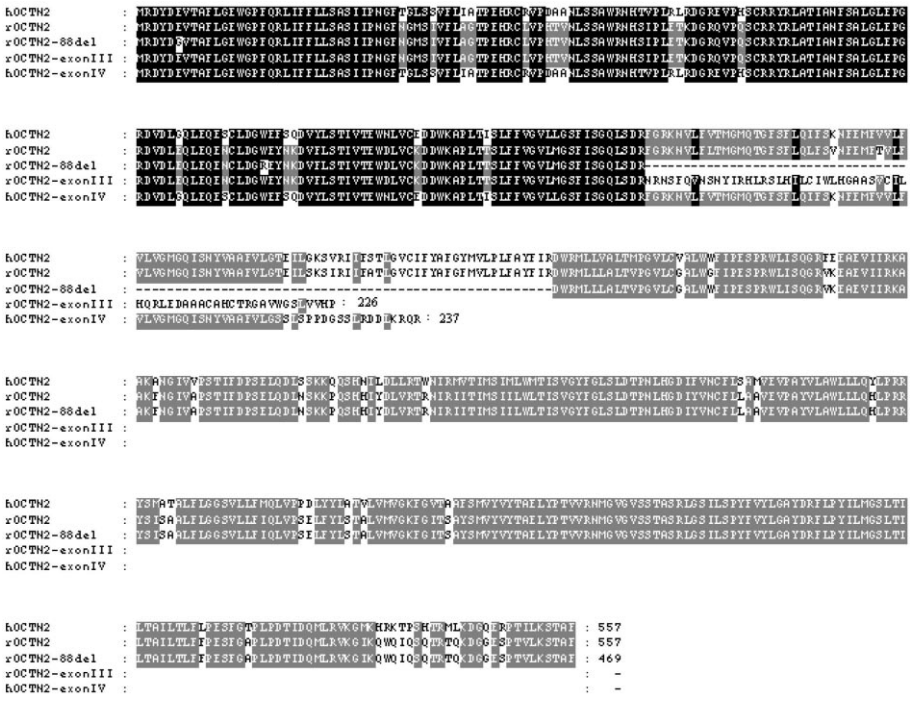

Fig. 3. Predicted amino acid sequence alignment of full length rat rOCTN2, the two splice variants reported in this study, human full length hOCTN2 and the exon IV splice mutation [20].

Using always the same forward primer and reporter probe we could vary the reverse primer to amplify only one specific transcript at a time. For the exon III reverse primer, only when exon III is completely spliced out and the start of exon IV joined to exon II can the primer anneal and transcription start. For the reverse primer −88del the same is true only for when exon III and part of exon IV are skipped and the primer can span the gap and serve as the initiation of transcription. The expression levels of each target cDNA was standardised first for each sample by the subtraction of the internal GAPDH standard and then made relative to each other by arbitrarily designating the full length transcript in liver as having 100% expression of the gene and all the others expressed as a percentage thereof. The efficiency of amplification was checked for all targets by performing a series of serial dilutions of template for each primer pair. The resulting quantitative PCR told us very clearly that OCTN2 was most abundant in the testis of rat, followed by liver and kidney tissues, however the heart and skeletal muscle, where carnitine concentrations are at their highest in humans, showed negligible amounts of OCTN2 transcript, neither full length nor either of the two variants (see Fig. 4). These results for the full length rat OCTN2 mRNA were well in accordance with the original data observed by Northern analysis when this transporter was first cloned by Sekine et al. [22]. Rat testis, next to epididymis, one of the highest carnitine storing tissues in rodents [2,1], showed the most significant amounts of both full length and variant mRNA's. Carnitine has long been shown to have a physiologically important role to play in the testis. It's high concentrations correlate directly with sperm maturation (as determined by motility and fertility) as they progress along the seminiferous tubules to the epididymis [27–31].

In all tissues tested, both splice variants could be detected and amplified with direct correlation to the level of starting template, though in low amounts relative to that of the full length sequence (Fig. 4). Again, only in testis was a significant level of expression detected for either of the splicing variants. The 88del variant, although accounting for only 1% of the total coding sequence showed a 10-fold higher level of expression than the other exon III variant, and two- to three-fold higher than that of the full length coding sequence found in low-expressing tissues such as heart and skeletal muscle.

Analysis of the predicted amino acid sequence [24,25] of the 88del splice variant reveals a protein with intact N and C termini with correct cytosolic orientation but missing four putative transmembrane domains after the extracellular loop (Fig. 5). The sugar transport signature is disrupted in both variants, sitting as it is on the exon II/III boundary. The exon III deletion, however, leaves a largely disrupted sequence, prematurely terminating the protein after the first two puta-

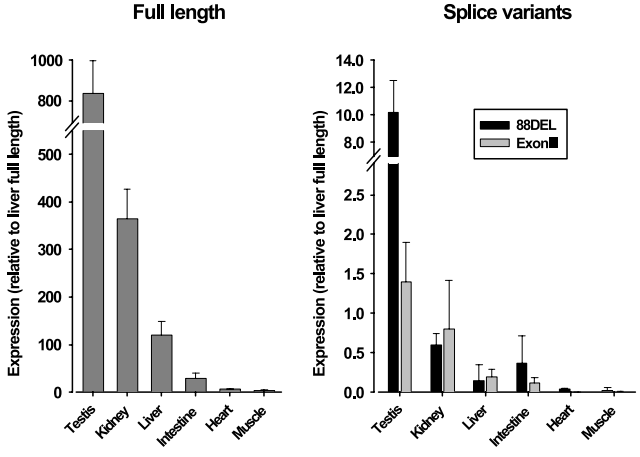

Fig. 4. Levels of mRNA transcripts in six different tissues of three rats were assessed for both the full length OCTN2 and both of the differentially spliced variants. Results were compiled from three animals, error is given as ±S.D.

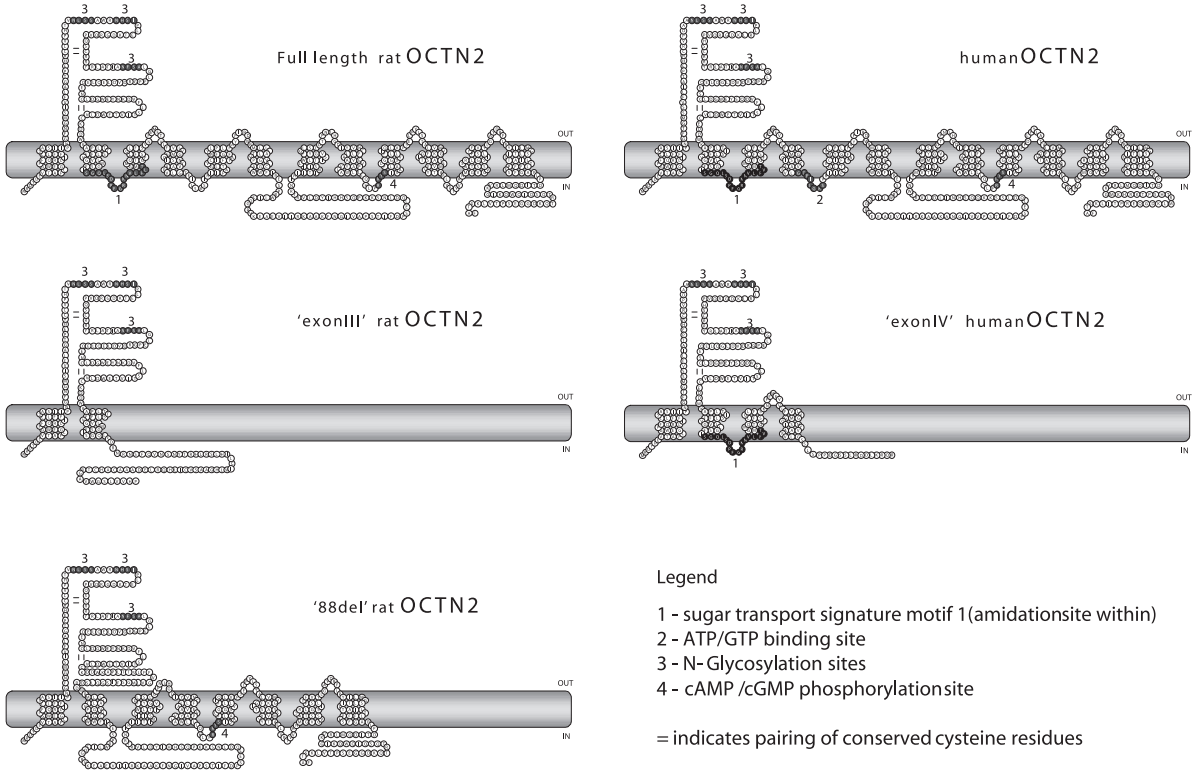

Fig. 5. Pictographical depiction of the protein products of all splice variants. '=' depicts possible interactions between N-terminal cysteine residues, highly conserved within the cation and anion transporter families.

tive transmembrane domains. The big extracellular loop remains with its glycosylation sites, along with two potential protein kinase C and one casein kinase 2 phosphorylation sites, and three out of seven myristolation sites still intact, indicating the potential to be still active depending on the mechanism of phosphorylation. However, the protein is minus its potentially important cAMP site and is only half the size of the parent transcript.

The splice variant 88del is missing 88 amino acids in the middle of the protein effectively omitting one third of its transmembrane segments. Unlike the exon III variant, it resumes its coding sequence in the original frame and so retains most of the C-terminal part of the protein and the inherent signature motifs including the cAMP phosphorylation site. However, similar to the exon III deletion, it has also lost its sugar motif and its amidation site.

The substrate binding sites of this Na^+ co-transporter of carnitine are unknown but the presence of four highly conserved cysteine residues within the N-terminal extracellular loop amongst all members of this family, and in some cases those of the anion transporter family as well, would indicate it's tertiary structure plays some functional role and its extracellular orientation would make it a likely candidate for the position of substrate recognition and/or binding. Retention of this loop but loss of any activation domains in a shortened version of the full length transporter, could play a role in regulation of transport by competition of the active and inactive forms at the cell surface. It would be of interest to note whether these variants are upregulated in response to altered extracellular carnitine levels.

A mutant transcript has been described in a patient with SCD in which exon IV has been deleted. Similar to our exon III, this leads to a resumption of an out-of-frame code and premature termination of the protein at 237 residues (19 amino acids after the splice site). Importantly for this splice mutation, the human amino acid sequence unlike the rat encodes an ATP/GTP binding motif at exactly this exon III/IV boundary. One amino acid difference in the rat renders this motif unrecognisable to a prediction server [25] as a potentially functional ATP/GTP binding domain. This would imply that the two proteins are most likely activated by different mechanisms of phosphorylation. Initial work by two independent research groups on human OCTN2 mRNA showed the presence of a double band in a Northern blot of adult kidney mRNA (absent in proximal tubules) [32,26] that was not detected in that of rat. Perhaps more relevant to this study, a second smaller transcript in the testis of rat was demonstrated by Northern blot in the original work which first cloned the gene [22].

In the observed cases to date for humans with SCD, there exist so far three known alterations of the splicing in hOCTN2.

One is clearly a mis-splicing event, a 19 bp intronic insertion which in turn, led to a frame shift and consequently a premature termination of the coding sequence. The second, as described by the same authors [20], is a clean exon IV deletion, though it was not described as such. Here, as in the intronic insertion above, the mis-splicing occurred before the premature stop, which resulted from the shift in the frame of the coding sequence. It should be mentioned that neither of these cases is appropriate to the mechanism of nonsense associated mRNA decay.

Nonsense associated alternative splicing (NAS) is part of the process of nonsense mediated mRNA decay (NMD)

which can involve both exon skipping and more often, intron retention. NMD, is thought to be a mechanism by which cells can eliminate imperfect mRNAs, which would lead to the translation of potentially harmful or toxic proteins.

The hallmark of NMD has been the reduction in abundance of the mRNA associated with a premature termination codon (PTC). In cases of NAS, the splicing occurs immediately after a PTC and is thought to provide a signal to mediate the rapid decay of the transcript.

The third case of mis-splicing reported for human OCTN2 was in a paper by Burwinkle et al. [21] who observed a PTC in exon V and then a skipping of exon VII in two unrelated German patients with SCD. One was heterozygous and both full length and shortened transcripts were observed, the homozygous RT-PCR produced only the shortened transcript. It was not indicated if any reduction in abundance of either transcript was observed, which would be indicative of NMD. However it is possible that what they observed was NAS, although the to-date speculated mechanism behind NAS would preclude the presence of the intervening exon VI, which came between the observed splice and the PTC. In all other cases of NMD/NAS observed so far the next exon/exon boundary is affected and seems to show a 'positional bias' i.e. if the PTC is within 50 bp 5′ of the exon boundary, in which case sometimes consecutive exons can be skipped [33–35].

OCTN2, in both humans and rats is clearly subject to splicing events. Here, we show the presence of two splicing variants in normal healthy male Sprague–Dawley rats.

We feel it is important to stress that we have not attempted to account for all splice variants of OCTN2, merely to point out that they exist for this gene and examine the abundance of those we knew existed. We think this would be interesting to follow up, especially in light of potential human splicing/mis-splicing events observed in patients with primary SCD.

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