Primary structure of rat plasma membrane Ca^{2+} -ATPase isoform 4 and analysis of alternative splicing patterns at splice site A

Timothy P. KEETON* and Gary E. SHULL†

Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Ave., ML 524, Cincinnati, OH 45267-0524, U.S.A.

We have determined the primary structure of the rat plasma membrane Ca^{2+} -ATPase isoform 4 (PMCA4), and have analysed its mRNA tissue distribution and alternative splicing patterns at splice site A. Rat PMCA4 (rPMCA4) genomic clones were isolated and used to determine the coding sequences and intron/exon organization of the 5'-end of the gene, and the remaining coding sequence was determined from PCR-amplified cDNA fragments. Pairwise comparisons reveal that the amino acid sequence of rPMCA4 has diverged substantially from those of rPMCA isoforms 1, 2 and 3 (73–76% identity) and from that of human PMCA4 (87%). Despite the high degree of sequence divergence between the two species, comparisons of intron and untranslated mRNA sequences with the corresponding human

sequences confirm the identity of this rat isoform as PMCA4. Northern blot studies demonstrate that the PMCA4 mRNA is expressed in all rat tissues examined except liver, with the highest levels in uterus and stomach. A combination of PCR analysis of alternative splicing patterns and sequence analysis of the gene demonstrate that a 36 nt exon at site A is included in PMCA4 mRNAs of most tissues but is largely excluded in heart and testis. Alternative splicing of both the 36 nt exon and a previously characterized 175 nt exon at splice site C, each of which can be either included or excluded in a highly tissue-specific manner, leads to the production of four different PMCA4 variants ranging in size from 1157 to 1203 amino acids.

INTRODUCTION

Fine control of intracellular Ca^{2+} concentrations in mammalian tissues is mediated primarily by calmodulin-sensitive plasma membrane Ca^{2+} -ATPases (PMCA). This family of Ca^{2+} pumps consists of approximately two dozen proteins encoded by the alternatively spliced transcripts of at least four genes, termed PMCA1, 2, 3 and 4. Full-length cDNAs encoding rat PMCA (rPMCA) isoforms 1, 2 and 3 [1,2], human PMCA (hPMCA) isoforms 1, 2 and 4 [3–5] and both pig [6] and rabbit [7] PMCA1 have been reported. Two regions of the PMCA primary transcripts, termed splice sites A and C, have been shown to be involved in alternative processing. Site A includes exons that encode residues in the vicinity of a phospholipid-responsive domain [8] located in the cytoplasmic loop between the second and third transmembrane domains. Splicing in this region has been demonstrated for rat isoforms 2 and 3 [9,10] and human isoforms 2, 3 and 4 [5,11]. Site C includes exons of the 3'-end of the PMCA genes which encode alternative calmodulin-binding domains and C-termini, and has been demonstrated to be involved in alternative splicing of the primary transcripts of the four known genes of both the hPMCA [11–13] and rPMCA [10,14].

The exact number of active PMCA genes present in the mammalian genome is uncertain. Partial sequences of the rat homologue of hPMCA4 [14,15] and the human homologue of rPMCA3 [11] have been reported, confirming the existence of at least four isoforms in both species. The sequence generated by reverse-transcription and PCR amplification of a rat kidney

PMCA mRNA by Gonzalez et al. [16] demonstrated a high degree of sequence divergence relative to rPMCA1, 2 and 3 and to hPMCA4, thereby raising the interesting possibility of a novel isoform expressed in kidney. An alternative explanation, however, is that the kidney cDNA encodes part of rPMCA4, which might exhibit greater sequence divergence between species than is observed for the other isoforms.

The present study was conducted in part to resolve these questions concerning the identity of PMCA isoforms in rat. To do this, we used a combination of genomic and PCR cloning techniques to obtain the complete coding sequence of rPMCA4, and confirmed its identity by comparing untranslated and intron sequences of the rat and human PMCA4 genes. This work demonstrates that the cDNA fragment cloned previously from rat kidney [16] was in fact derived from the rPMCA4 mRNA and that there is extensive amino acid sequence divergence between the rat and human enzymes. A second reason for cloning rPMCA4 is that it allows us to complete the characterization of the known PMCA isoforms in the rat with respect to their primary structures, alternative splicing patterns and mRNA tissue distributions. This is important because the rat is the most commonly used model for studies of the physiological function and regulation of the plasma membrane Ca^{2+} pumps in animal tissues [17–22]. Our analyses of the rPMCA4 mRNA tissue distribution and alternative splicing patterns demonstrate that there are substantial differences between the expression patterns of PMCA4 in rat and those determined recently for humans [11], and indicate that the biological role of PMCA4 may be more complex than is suggested by its general designation as a housekeeping enzyme.

Abbreviation used: r/hPMCA, rat or human plasma membrane Ca^{2+} -ATPase.

The nucleotide sequence data reported in this paper will appear in the GenBank and EMBL Nucleotide Sequence Databases under the accession number U15408.

* Pre-doctoral Fellow of the Albert J. Ryan Foundation. Current address: Department of Molecular Biology, University of Wyoming, Laramie, WY 82071-3944, U.S.A.

† To whom correspondence should be addressed.

MATERIALS AND METHODS

Isolation and analysis of genomic clones

A rat genomic library was screened using a ^{32}P -labelled PCR product corresponding to nts -26 to 249 of the hPMCA4 cDNA [4] as a probe, and a cosmid clone (4-34a) containing coding sequences from the 5'-end of the rPMCA4 gene was isolated. Two *Eco*RI fragments of clone 4-34a, 9 and 1.4 kb in length and containing the 5'-most coding exons, were identified by Southern blot analysis using the same probe that was used for screening the library. A 3 kb *Eco*RI fragment containing the 36 nt exon at splice site A was identified using a ^{32}P -labelled PCR product corresponding to rPMCA4 nts 621 to 1042 (see Figure 2). These *Eco*RI restriction fragments were subcloned into the plasmid pBluescript. The 9 kb *Eco*RI fragment was also digested with *Sau*3A1, shotgun subcloned into pBluescript and a subclone containing sequences from the 5' untranslated region and terminating at a *Sau*3A1 site at nt 132 was identified by colony hybridization. Sequence analysis was performed with the Sequenase II protocol (United States Biochemical) using double-stranded plasmid DNA as template.

Northern blot hybridization analysis

The rat Northern blot was prepared as described previously [2] with each lane containing 5 μg of poly(A)⁺ RNA from the tissues indicated. Hybridization was performed in 50% formamide, 5 $\times$ SSPE, 2 $\times$ Denhardt's solution, 0.1% SDS and 100 μg of denatured salmon sperm DNA per ml, at 43 $^{\circ}\text{C}$ for 14 h. The probe cocktail consisted of 50 ng of an equimolar mixture of three PCR products corresponding to nts -123 to 216, 1455 to 1961 and 3394 to 3901 of rPMCA4a that had been labelled using oligonucleotide primers corresponding to the 5'-end of the non-coding strand of each product. [α - ^{32}P]dCTP was incorporated into the probe using the Klenow fragment of DNA polymerase I (GIBCO-BRL) in a buffer consisting of 50 mM Tris (pH 7.5), 10 mM MgCl_2 and 1 mM dithiothreitol. The blot was washed once for 45 min at room temperature in 2 $\times$ SSC/0.5% SDS, once for 45 min at 50 $^{\circ}\text{C}$ in 2 $\times$ SSC/0.5% SDS and once for 45 min at 60 $^{\circ}\text{C}$ in 1 $\times$ SSC/0.5% SDS. (For the composition of Denhardt's solution, SSC and SSPE, see [23].) The blot was then analysed by autoradiography using Kodak X-Omat film at -80 $^{\circ}\text{C}$ with an intensifying screen for 7 days.

PCR amplification of rPMCA4 cDNA clones

In order to fill in the 5'-most gap in the coding sequence obtained from subclones of cosmid 4-34a, a 400 bp PCR product was amplified from testis poly(A)⁺ RNA which had been reverse transcribed with Superscript reverse transcriptase (GIBCO-BRL) using random hexamers as primers. The specific primers for this PCR amplification, which was carried out essentially as described previously [14], corresponded to nts 80 to 103 (sense strand) and 456 to 479 (antisense strand) of the rPMCA4 cDNA and included *Not*I restriction endonuclease recognition sequences in the 5'-ends. The resulting product was cut with *Not*I, subcloned into pBluescript and sequenced using primers corresponding to the T7 and T3 RNA polymerase promoters in the vector. The sequence corresponding to nts 2931 to 3249 was obtained from a 360 nt PCR product spanning codons 970 to 1089 that was prepared in our previous study of splicing patterns at site C [14]. The main portion of the coding sequence was provided by a 2.3 kb PCR product amplified from reverse-transcribed rat testis poly(A)⁺ RNA. The primers for this reaction corresponded to rPMCA4 nts 621 to 641 (sense strand) and 2932 to 2952 (antisense strand). In order to detect any mutations introduced

by the thermostable polymerase, three separate clones were isolated, subcloned, and sequenced using specific primers. Taq polymerase (GIBCO-BRL) was used for all PCR amplifications in this study.

PCR analysis alternative splicing at site A

For PCR analysis of splicing at site A, first strand cDNA was prepared as described previously [14]. Following an initial amplification of 30 cycles, 10% of each 100 μl PCR reaction was used as template for ten additional cycles of amplification in a fresh reaction mixture. Of this final round, 15% was run in a 1.5% agarose gel, photographed, and Southern blotted onto Nytran membranes (Schleicher & Schuell). The primers used for this study corresponded to rPMCA4 nts 621 to 641 (sense strand) and 1025 to 1042 (antisense strand) and the resulting 385 and 421 bp products were purified, subcloned into pBluescript, and sequenced. Blots were hybridized with a 5'-end-labelled oligonucleotide corresponding to nts 952-972 for 8 h at 55 $^{\circ}\text{C}$ in 6 $\times$ SSC, 1 $\times$ Denhardt's solution, 0.1% SDS and 100 μg of denatured salmon sperm DNA per ml and washed as described previously [14].

RESULTS

Cloning of rPMCA4 genomic and cDNA clones

Full-length rat cDNAs have been isolated and characterized for PMCA isoforms 1, 2 and 3 [1,2] but only partial sequences have been determined for rPMCA4 [14,15]. Also, the intron/exon organization at splice site C has been determined for all four PMCA genes, but the organization at splice site A has been determined only for isoforms 1, 2 and 3 and is lacking for PMCA4. Our strategy (see Figure 1) for obtaining the complete coding sequence of this isoform and for determining the genomic organization at splice site A was to isolate genomic clones containing the 5'-end of the rPMCA4 gene, determine the intron/exon organization of this region and then use primers based on the sequence from the 5'-most coding exons and the previously determined sequence from the 3'-end [14] to perform reverse transcription and PCR amplification of the rPMCA4 mRNA.

Screening of a rat genomic library with a 275 bp PCR product corresponding to the 5'-end of the hPMCA4 cDNA provided a single cosmid, clone 4-34a, with a 13.4 kb insert containing *Eco*RI fragments that were 1.4, 9, and 3 kb in length. Southern blot analysis showed that the 275 bp probe used in screening the library hybridized with the 1.4 and 9 kb *Eco*RI fragments, and later analysis showed that the 3 kb fragment contained the alternatively spliced exon at splice site A (discussed below). Each of these *Eco*RI fragments, as well as a *Sau*3A1 fragment from the 9 kb *Eco*RI fragment, was subcloned and used to determine the

Figure 1 Schematic diagram of the rPMCA4 mRNA and subclones used for sequence analysis

The middle bar represents the PMCA4 mRNA with untranslated regions indicated by dark areas and coding sequences represented by open areas. Alternative splice sites A and C are labelled. Sequences derived from PCR products are indicated by hatched bars and sequences derived from genomic fragments are indicated by stippled bars. The scale is in kb.

(a) 5'-Untranslated sequence

(b) Site C introns

(c) 3'-Untranslated sequence

Figure 3 Comparison of untranslated and intron sequences of rat and human PMCA4

Rat nucleotide sequence is shown on top with the corresponding human sequence (from [4] and [12]) below, and amino acids are shown above or below the coding sequences. Nucleotide identity is represented by a dash in the human sequence and gaps added to align the sequences are represented by carets. (a) The sequence of the 5' untranslated region and the first four codons of the coding sequence. (b) Intron sequences flanking the three exons involved in alternative splicing of the 175 nt exon at site C. Exons are boxed and the beginning and end of introns are overlined. (c) The sequence of the PMCA4b 3' untranslated region and last three codons.

Given that Taq DNA polymerase is known to miss-incorporate nucleotides at a low frequency, three independent isolates were sequenced for each PCR product. The only inconsistencies noted were in one of the 2.3 kb isolates, which contained three nucleotide changes that altered a codon relative to the other two isolates. These alterations (Glu-236 → Gly, Asp-385 → Gly and Met-802 → Ile) differed from the corresponding residues of hPMCA4, whereas the residues occurring in the two isolates that were consistent matched those of hPMCA4. It is therefore likely that these sequence variations are the result of Taq polymerase replication errors. In combination with sequences from the 5'- and 3'-ends of the cosmid clones, the PCR clones provided the entire rPMCA4 coding sequence which is shown along with the deduced amino acid sequence in Figure 2. The reading frame indicated encodes a protein with a molecular mass of 127400–133100 Da, depending upon the use of alternatively spliced exons at sites A and C (boldface sequences, Figure 2). As discussed below, each of these exons can be either included or excluded, with all four combinations being utilized.

Table 1 Percentage amino acid identity in pairwise comparisons of PMCA isoforms

Comparisons are between rat and human PMCA isoform variants which contain both the single-exon insertion at site A and the exon at site C that encodes the acidic calmodulin-binding domain.

Isoform	Amino acid identity (%)					
rPMCA1	—	—	—	—	—	—
rPMCA2	81	—	—	—	—	—
rPMCA3	82	80	—	—	—	—
rPMCA4	76	73	75	—	—	—
hPMCA1	99	83	80	75	—	—
hPMCA2	79	99	83	73	81	—
hPMCA4	76	75	76	87	76	73
	r1	r2	r3	r4	h1	h2

Confirmation of identity

The enzyme cloned above is clearly a member of the plasma membrane Ca^{2+} -ATPase family, however, as discussed below, its amino acid sequence exhibits a greater degree of divergence from that of hPMCA4 than observed in cross-species comparisons of the other isoforms. To confirm that it is the rat homologue of hPMCA4 rather than a novel isoform, we performed comparisons (Figure 3) between the published untranslated [4] and intron sequences [12] of hPMCA4 and the corresponding sequences of the rat gene, which we determined from subclones of genomic cosmid clones isolated in this study and in a previous study [14]. The 5' and 3' untranslated sequences exhibit 83% and 69% nucleotide sequence identity respectively, and the intron sequences flanking the alternatively spliced 175 nt exon at site C [14] exhibit 75% nucleotide identity. Overall sequence identity for the 586 non-coding nucleotides compared between rats and humans was 76%. Based on the high degree of nucleotide sequence identity observed in these non-coding regions we conclude that the sequence reported in this work is that of rPMCA4.

Amino acid sequence comparisons

Table 1 lists the percentage of amino acid identities observed in pairwise comparisons between PMCA isoforms from rats and humans. For each isoform the variant being compared is the one containing both the alternative exon sequence at splice site A (the 'x' splicing option [9,11]) and the exon encoding the acidic variable region of the calmodulin binding domain at splice site C (the 'a' splicing option [14]). Rat isoforms 1, 2 and 3 and human isoforms 1 and 2 exhibit 80–82% amino acid identity with one another and 99% identity is observed in cross-species comparisons of rat isoforms 1 and 2 with their human homologues. Rat and human PMCA4 exhibit 73–76% amino acid identity with the other isoforms and they exhibit only 87% identity with each other. Thus, among the four isoforms, PMCA4 is the most divergent.

A comparison of the rPMCA4 and hPMCA4 amino acid sequences is shown in Figure 4. From the N-terminus through to the constant region of the calmodulin-binding domain (amino acids 1 to 1103) the two proteins align perfectly and exhibit 88% amino acid identity. In general, sequence divergence occurs throughout this region and is approximately the same in the transmembrane domains as it is in the cytoplasmic regions. The extreme N-terminal sequence and the short extracellular junction between the first and second transmembrane domains shows less

Figure 4 Amino acid sequence comparison of rat and human PMCA4

rPMCA4 amino acids are shown above the corresponding human sequence and are numbered on the right. Amino acid identity between the rat and human isoforms is represented by a dash in the human sequence. Boxed areas represent potential transmembrane domains and are labelled H1, H2, etc. The phosphorylation site (PHOS SITE) is indicated. The sequence encoded by the alternative exon at site A is in a dashed box and is labelled 'A'. Constant and variable regions of the calmodulin-binding domain are overlined and labelled CBD-C and CBD-V respectively. The amino acid sequence labelled PMCA4a in the box at the bottom is encoded by the mRNA which includes the 175 nt site C exon (or 178 nt in human); the PMCA4b sequence at the bottom is produced by the removal of this exon. Carets represent gaps introduced to maintain the alignment.

than the average degree of similarity, whereas the cytoplasmic sequences flanking the fourth and fifth transmembrane domains, which are involved in catalytic functions, are highly conserved. The C-terminal region, beginning with the variable region of the calmodulin-binding domain, exhibits considerably more divergence than the rest of the protein, which is surprising in the light of its known regulatory functions. This region of rat and human PMCA4a, which is produced by the inclusion of a 175 or 178 bp exon respectively, exhibits 72% amino acid identity. The corresponding regions of rat and human PMCA4b are only 51% identical, with multiple gaps required in order to maintain the alignment.

Northern blot analysis of rPMCA4 mRNA tissue distribution

In a previous study [14], we screened a Northern blot of poly(A)⁺ RNAs from adult rat tissues using a 275 bp probe corresponding to the 5' untranslated sequence and the beginning of the coding sequence of hPMCA4, and detected an approx. 8.5 kb mRNA in all tissues except liver. In a more recent study using PCR techniques [12], PMCA4 mRNA was detected in all human tissues examined, including liver. Because many of the signals observed in our previous study were faint it seemed possible that the PMCA4 mRNA was present in rat liver, but at levels below the limits of detection when using the short human PMCA4 probe, which was only 82% identical with the rat sequence. Thus, we have repeated this analysis using rat cDNA probes.

Both the size of the rPMCA4 mRNA and the pattern of expression (Figure 5) are the same as those determined previously with the human probe, although the signal intensities are much greater due to the use of a longer species-specific probe. The approximately 8.5 kb PMCA4 mRNA was not detected in liver

Figure 5 Northern blot hybridization analysis of rPMCA4 mRNA expression

Poly(A)⁺ RNA from the indicated tissues was analysed by Northern blot hybridization using a cocktail of rPMCA4 cDNA probes as described in the Materials and methods section. The positions and sizes (in kb) of the RNA markers are indicated on the right.

but was detected in all other tissues. It was expressed at high levels in uterus and stomach, at intermediate levels in brain, lung, testis, small intestine and large intestine and at low levels in skeletal muscle, heart, kidney and spleen. Spleen also contained a very low abundance 7 kb mRNA that hybridized with the probe.

PCR analysis of splice site A

Alternative splicing at site A, located just 5' to the sequence encoding an apparent phospholipid binding domain [8,25], has been demonstrated in r/hPMCA2 [5,9], rPMCA3 [10] and hPMCA4 [11]. However, detailed studies of the site A splicing patterns in rPMCA4 have not been reported nor has the intron/exon organization of this region of the PMCA4 gene been examined. To investigate tissue-specific splicing patterns at site A, we performed reverse transcription and PCR amplification of poly(A)⁺ RNA from the same rat tissues used in our Northern

Figure 6 PCR analysis of alternatively spliced rPMCA4 mRNAs at site 'A'

Poly(A)⁺ RNA from each of the indicated rat tissues was reverse transcribed and used for PCR analysis as described in the Materials and methods section. Upper left, the products of the PCR reaction separated in an agarose gel and stained with ethidium bromide. Upper right, Southern blot analysis of the same gel shown on the left using a 5'-end-labelled internal oligonucleotide as probe. Bottom panel, deduced amino acid sequence of alternatively spliced mRNAs obtained by sequence analysis of each PCR product. The numbers on the left indicate the size (in bp) of the corresponding PCR products.

Figure 7 Alternative splicing patterns involving a 36 nt exon at site A of the rPMCA4 gene

The diagram illustrates the two splicing options available in this region of the primary transcript. The exon sequence is boxed and shown in upper case letters and the flanking intron sequences are shown in lower case letters with splice donor and acceptor sites underlined. Amino acids encoded by the exon sequence are shown below the nucleotide sequence.

blot analyses. A 421 bp product corresponding to nts 621 to 1042 was detected in all tissues, however in heart and testis a more abundant 385 bp product was also detected (Figure 6). Sequence analysis revealed that the smaller product lacks 36 nts, corresponding to positions 902 to 937, which were present in the longer product. Additional analysis of the rPMCA4 gene demonstrated that the 36 nt sequence is a single exon flanked by consensus acceptor and donor splice sites (Figure 7).

DISCUSSION

With this report of the rPMCA4 cDNA sequence we have completed the determination of the primary structures of the four known PMCA isoforms in the rat. In addition, we have carried out Northern hybridization analysis of the distribution of PMCA4 mRNAs in adult rat tissues and PCR analysis of the alternative splicing patterns at site A, which can be directly compared with our previous analyses of the tissue distribution of mRNAs encoding rat PMCA1, 2 and 3 [2] and splicing patterns for all four isoforms at site C [14].

Among the four known isoforms, PMCA4 exhibits the greatest divergence in amino acid sequence. It is 73–76% identical with the other isoforms and in cross-species comparisons between human and rat it exhibits only 87% amino acid identity. Although this degree of similarity is low compared with isoforms 1 and 2, which are 99% identical between human and rat, it is

not unusual. For example, the rat and human cystic fibrosis transmembrane conductance regulators are only 79% identical [26] and the gastric H,K-ATPase β -subunits of rat and rabbit [27,28] are only 82% identical. Also, multiple gaps were required to align these proteins, whereas rat and human PMCA4 aligned perfectly for over 1100 amino acids and required gaps only to align the C-terminal regulatory regions. Nevertheless, we considered it necessary to confirm the identity of rPMCA4 and to do this we compared the untranslated sequences and intron sequences of the rat and human genes. The high degree of sequence similarity in these non-coding regions confirmed that the enzyme we have cloned is the rat homologue of hPMCA4, and further confirmation was provided by the observation that the same Northern hybridization pattern was obtained with the rat probe (Figure 5) as seen previously with a hPMCA4 probe [14].

A cDNA fragment encoding part of a PMCA isoform that seemed to differ from the four known isoforms was recently cloned by PCR amplification of poly(A)⁺ RNA from rat kidney [16]. However, the partially deduced amino acid sequence of the kidney cDNA is virtually identical with that of rPMCA4 cloned in the present study. The only differences are valines at positions 602 and 687 determined in the earlier study [16] rather than aspartic acid and alanine respectively that were determined in the current study; the corresponding residues in hPMCA4 are asparagine and alanine [4]. Thus, we conclude that the cDNA fragment cloned by Gonzalez et al. [16] was derived from the PMCA4 mRNA rather than from a fifth isoform.

The greatest degree of sequence divergence between human and rat PMCA4 occurs in the C-terminal regulatory domains. This is particularly apparent for the PMCA4b C-terminus in which there are many non-conservative changes, multiple gaps and only 51% amino acid identity. In contrast, almost all of the amino acid differences between the C-termini of rat and human PMCA4a are conservative. However, an interesting and potentially significant species difference in the variable region of the calmodulin-binding domain of PMCA4a is the replacement of Thr-1109 in humans with lysine in the rat, which would make the calmodulin-binding domain of this variant less acidic. Based on studies from Enyedi et al. [29] such a change would be expected to increase its affinity for calmodulin, thereby leading to an increase in its apparent affinity for Ca²⁺. Other sequence differences which could lead to alterations in functional properties

are the occurrence of histidine residues in one species or the other. hPMCA4a contains a histidine at position 1128 which is replaced by a glutamine in rat and variations in the presence of histidine residues in PMCA4b occur at positions 1115, 1118, 1132, 1183 and 1191 (in the rat sequence). Kessler et al. have shown that the presence or absence of histidine residues in alternatively spliced C-terminal regions of PMCA1 is correlated with alterations in pH sensitivity of calmodulin binding [30]. It is therefore possible that these changes lead to species differences in the pH optima for calmodulin binding.

In general, the results of our PMCA4 tissue-distribution studies and analysis of splicing patterns at site A in the rat are similar to those obtained in a previous study of hPMCA4 [11], although there are some significant species differences. Using Northern blot analysis, we detected PMCA4 mRNA in all rat tissues examined except liver and the levels of expression in different tissues varied greatly. It should be noted that the lack of a PMCA4 hybridization signal in liver is not due to the quality of the RNA, as the blot used in this study is the same blot that was used in our previous analysis of PMCA1 mRNAs (see Figure 5 of [2]), which gave a clear signal in liver. In contrast, using quantitative PCR analysis, PMCA4 mRNA was detected at relatively uniform levels in a variety of human tissues, including liver, and there was little difference between the amounts of PMCA4 mRNA measured in liver, kidney, lung and stomach [11].

Like the rat, splicing at site A in hPMCA4 involves the inclusion or exclusion of a single 36 nt exon to yield the 'z' or 'x' splice variants respectively [11]. This exon was included in PMCA4 mRNAs in all human tissues examined, except heart where it was excluded in about half of the mRNA [11]. Rat heart also expresses the variant lacking the 36 nt exon but it is the predominant form and very little mRNA containing this exon is present. The predominant form in rat testis also lacked this exon but there is no corresponding information about expression in human testis.

Based on the tissue distributions and relative amounts of the two site A splice variants (Figure 6) and the two site C splice variants analysed previously [14], it is apparent that all four possible combinations are utilized in the generation of PMCA4 variants. Site A splicing in brain and lung was exclusively the 'x' option (containing the 36 nt exon), but at site C [14] most of the mRNA in brain utilized the 'a' splice option (including the 175 nt exon) and most of the lung mRNA utilized the 'b' splice option. The resulting PMCA4xa and 4xb variants would contain 1169 and 1203 amino acids respectively. Site A splicing in heart and testis was predominantly the 'z' option (lacking the 36 nt exon), but in testis, site C splicing was predominantly the 'a' option and in heart it was predominantly the 'b' option. These 4za and 4zb variants would contain 1157 and 1191 amino acids, respectively.

Because it seems to be expressed at relatively uniform levels in all human tissues, it has been suggested that PMCA4 may serve a housekeeping function [11]. However, the results of the current study, showing substantial variations in PMCA4 mRNA levels in adult rat tissues, indicate that its function may be more complex. This possibility is supported by recent *in situ* hybridization analyses of PMCA4 mRNA distribution in rat brain [31]. Rather than being expressed at uniform levels

throughout the brain, as might be expected for a housekeeping enzyme, it exhibited high levels of expression in piriform cortex and regions of the neocortex and seemed to be absent or present at reduced levels in other regions. Thus, the available information suggests that variants of PMCA4, at least in rat, might be involved in more specialized functions.

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