Analysis of the Tissue-specific Distribution of mRNAs Encoding the Plasma Membrane Calcium-pumping ATPases and Characterization of an Alternately Spliced Form of PMCA4 at the cDNA and Genomic Levels

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The plasma membrane Ca\(^{2+}\)-pumping ATPase (Ca\(^{2+}\)-ATPase) mRNAs are encoded on four different genes designated PMCA1–PMCA4. The primary transcripts from some of these genes are known to be alternately spliced in the region encoding the regulatory domains of the enzymes. The known alternately spliced forms of these Ca\(^{2+}\)-ATPase mRNAs and a new spliced variant of PMCA4 (PMCA4b), presented here, represent at least nine different mRNAs encoding the Ca\(^{2+}\)-ATPases. In this report, the examination of the tissue-specific distribution of these alternately spliced mRNAs using polymerase chain reaction amplification of cDNA coupled with Southern blotting revealed that each spliced variant had a unique tissue distribution. PMCA1a and PMCA4a were present in all tissues examined. PMCA1a, PMCA1b, and PMCA4b were expressed in excitable tissues, whereas PMCA1d was expressed only in muscle tissues. PMCA2 was found in liver, adrenal gland, spinal cord, and brain. PMCA3a was present in spinal cord, and PMCA3b in thymus, adrenal gland, spinal cord, and brain.

The mRNA for a new spliced variant of PMCA4 (PMCA4b) was detected in this study. Complementary DNAs for this isoform were isolated and characterized from human and bovine brain. This alternately spliced form of the PMCA4 mRNA contained an exon inserted at the splice junction immediately following the sequence encoding the calmodulin-binding domain. As has also been shown for PMCA1a, this insertion produced a shift in the reading frame at the 3'-end of the PMCA4 mRNA that yielded a sequence encoding a Ca\(^{2+}\)-ATPase lacking a large portion of the C-terminal regulatory domain. When the human PMCA4 gene spanning this region of variable exon splicing was sequenced, it confirmed the intron-exon boundaries where alternate splicing occurs to produce PMCA4a and PMCA4b.

The plasma membrane calcium-pumping ATPase (Ca\(^{2+}\)-ATPase) regulates cytosolic calcium concentrations in all cells by extruding calcium ions in an ATP-dependent manner. The Ca\(^{2+}\)-ATPase continues to remove calcium from the cell until the free cytosolic concentration drops below 10\(^{-7}\) M. This final cytosolic calcium concentration is below the \(K_{d(Ca^{2+})}\) for calmodulin, so the many cellular processes regulated by the Ca\(^{2+}\)-calmodulin complex are affected.

The activity of the plasma membrane Ca\(^{2+}\)-ATPase is modified by a large number of agents that interact either with the enzyme directly or through some integrated regulatory pathway. The enzyme found in human erythrocytes is stimulated by Ca\(^{2+}\)-calmodulin (Bond and Clough, 1973), calbindin-9K (Walter, 1989), phosphorylation by cAMP-dependent kinase (Neyses et al., 1985), protein kinase C (Neyses et al., 1988), thyroid hormone (Galo et al., 1981), acidic phospholipids (Carafoli and Zurini, 1982), mild proteolysis (Zurini et al., 1984; Sarkadi et al., 1986; James et al., 1989), and self-association (Kosk-Kosicka and Bzdenga, 1988). Oxytocin depresses ~43% of the Ca\(^{2+}\)-ATPase activity in myometrium and ~15% in fat pad adipocytes, but has no effect on the enzyme activity found in duodenum (Soloff and Sweet, 1982).

In adipocytes, insulin causes the Ca\(^{2+}\)-ATPase to be less active; but in liver plasma membrane preparations, it has no effect (Lotersztajn et al., 1985). In isolated primary hepatocytes and membranes isolated from whole livers perfused with vasopressin, phenylephrine, epinephrine, or angiotensin II, the free cytosolic concentration drops below 10\(^{-7}\) M. This final cytosolic calcium concentration is below the \(K_{d(Ca^{2+})}\) for calmodulin, so the many cellular processes regulated by the Ca\(^{2+}\)-calmodulin complex are affected.

For the cases where it is known, these agents appear to exert their regulatory effects by interaction with a region near the carboxyl terminus of the enzyme. The calmodulin-binding site is in the carboxyl-terminal 9 kDa of the Ca\(^{2+}\)-ATPase (Zurini et al., 1984). Cleavage in this 9-kDa region by trypsin, chymotrypsin, and calpain activates the enzyme (Zurini et al., 1984; Sarkadi et al., 1986; James et al., 1989). Calbindin-9K protein has been shown to interact with the calmodulin-binding site in this 9-kDa region of the enzyme (James et al., 1991). The CAMP-dependent protein kinase and protein kinase C phosphorylation sites also have been found in this 9-kDa region (James et al., 1988; Wang et al., 1991).

Recently, several cDNA clones representing the coding region of the Ca\(^{2+}\)-ATPases have been isolated (Brandt et al., 1988; Shull and Greeb, 1988; Verma et al., 1988; Greeb and Shull, 1989; Strehler et al., 1990). It appears that there are four families of Ca\(^{2+}\)-ATPase mRNAs, each derived from a...
unique gene. The primary transcript from each gene can be alternately spliced in the region encoding the regulatory domain, which may lead to changes in the primary structure of these mRNAs results in alterations in the primary structure of the enzyme at the genetic level. The variations in the response of the Ca2+-ATPase to different agonists and antagonists. Since these effectors modify the Ca2+-ATPase activity differently to the PMCA3 isoform mRNA, except that the oligonucleotide e. This primer was complementary to positions 3421-3452 in hPMCAlb (human) (Verma et al., 1988), 3242-3272 in rPMCA3 (Greeb and Shull, 1989), and 3379-3410 in rPMCA2 (rat) (Shull and Greeb, 1988). Thus, the variations in the response of the Ca2+-ATPase to the effectors discussed above may be a reflection of the great diversity of the enzyme at the genetic level.

Since these effectors modify the Ca2+-ATPase activity differently in different tissues and cell types, it is possible that the mRNAs for the Ca2+-ATPases may be differently expressed in response to the metabolic or regulatory use of Ca2+ by particular cells and tissues. For this reason, we elected to examine the tissue distribution of Ca2+-ATPase mRNAs.

We chose to utilize a very sensitive and selective PCR-based mRNA amplification method coupled with Southern blotting. This method detected several possible novel spliced forms of the PMCA mRNAs. We have isolated cDNA for one of these new spliced forms of the PMCA4 mRNA. This new isoform mRNA (designated PMCA4b) contained an additional segment in the region encoding the regulatory domain. We present here the isolation of cDNAs from human and bovine brain and human genomic DNA sequences for the PMCA4b mRNA. Thus, the variations in the response of the enzyme to different agonists and antagonists.

FIG. 1. Possible exon combinations for each Ca2+-ATPase mRNA. The exon combinations of the various Ca2+-ATPase mRNAs that occur in the region near the end of the coding sequence are shown. These exon combinations are based on isolated cDNA sequences, except for PMCA3b, which was hypothesized by Greeb and Shull (1989). The expected sizes of the PCR amplification products from each of these different mRNAs are shown at the right. 4 Shull and Greeb (1988); 5 Verma et al. (1988); 6 Streherl et al. (1989); 7 Greeb and Shull (1989); 8 Streherl et al. (1990); 9 this study.

The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); SDS, sodium dodecyl sulfate.
Ca2+-ATPase isoforms unambiguously because of the low abundance of their mRNAs.

Southern Blotting of PCR Products—Fifteen microliters of the PCR products were resolved on a 3% composite agarose gel (2% NuSieve low melting agarose, FMC, Rockland, ME) and 1% medium EEO agarose (Type II, Sigma) in the presence of 0.5 µg/ml ethidium bromide. The resolved DNA fragments were denatured by soaking the gel in 1 M NaOH for 5 min, followed by neutralization in 1 M Tris-HCl, 3 M NaCl, pH 7.5, for 5 min. The DNA was then transferred to a nylon membrane (Hybond-N) in 20 M NaOH for 5 min, followed by neutralization in 1 M sodium chloride, 15 mM sodium citrate, pH 7.0 overnight. The DNA was fixed to the membranes by cross-linking with 300 nm light for 3 min.

Before a membrane was probed with a different oligonucleotide, the previous probe was stripped from the filter by incubation in 0.2 M NaOH at 65 °C for 1-2 h. The removal of the probe from the nylon membrane was monitored by autoradiography.

FIG. 2. Summary of PMCA4b DNA inserts and their positions relative to each other. The relative positions of bovine and human brain cDNAs of the plasma membrane Ca2+-ATPase isoform PMCA4b are shown. CAATPl, BB-4, and BB-6 were isolated from bovine brain cDNA libraries. HB-4 was isolated from a human fetal brain cDNA library. Oligo A and Oligo B refer to oligonucleotides that were used to prime first-strand cDNA syntheses for libraries constructed by primer extension. Their sequences and positions are described in the text. The arrows underneath the clones represent the strategy that was employed to sequence the cDNAs. The positions of exons A–C are shown at the top. The 5’-end of exon A and the 3’-end of exon C have not been determined and are so indicated. E, EcoRI; H, HindIII; Hn, HincII; N, NsiI; S, SacI.

Since the isolation of the cDNA sequence designated CAATPl (Brandt et al., 1988), a new nomenclature for plasma membrane Ca2+-ATPases has been adopted (Greeb and Shull, 1989). In the new nomenclature, cDNAs related to CAATPl are referred to as PMCA1. Throughout this manuscript, the new nomenclature has been adopted, except where a direct reference to the cDNA sequence published by Brandt et al. (1988) is necessary.
1356 in the insert of a recombinant bacteriophage isolated from the bovine brain cDNA library constructed as described above (BB-4) (Fig. 2). The DNA was made double-stranded and blunt-ended as described above. At this point, a blunt-to-EcoRI adaptor was ligated onto the cDNA. The adaptor consisted of the two oligonucleotides 5'-CTCGTGGCG-3' (oligonucleotide 1) and 3'-GAGCACCGCT-TAA-5' (oligonucleotide 2). Oligonucleotide 1 was first phosphorylated with polynucleotide kinase and ATP (Tabor, 1987). After the kinase reaction was completed, the polynucleotide kinase was inactivated by incubation at 55 °C for 5 min. Equimolar amounts of phosphorylated oligonucleotide 1 and nonphosphorylated oligonucleotide 2 were added to the blunt-ended cDNA at a 100-fold molar excess and ligated with T4 DNA ligase. The ligation products were chromatographed on Sepharose CL-4B, precipitated with sodium acetate and ethanol, and dissolved in 20 μl of water. The 5'-end of the EcoRI site was phosphorylated with polynucleotide kinase and ATP. The cDNA was extracted with 1 volume of saturated phenol and 1 volume of chloroform and precipitated with ethanol in the presence of ammonium acetate. The cDNA was then ligated into the vector λgt10 and processed as described above.

Screening of cDNA Libraries—The first bovine brain cDNA library was screened with an EcoRI/SacI fragment from positions 1 to 597 in the cDNA sequence of CAATP1 (Brandt et al., 1988). The second bovine brain cDNA library and a human fetal brain cDNA library (Neve et al., 1986) were screened with the upstream EcoRI fragment isolated from the insert of Xβ-4 (Fig. 2). The screening techniques were as described by Maniatis et al. (1982).

Isolation of PMCA4 DNA-containing Phage from Human Genomic Library—The homologous recombination system of Seed (1983) was used to isolate human genomic clones, with the exception that the Xα7N was used instead of +Xβ, and E. coli MC1061[p3] was used instead of E. coli W3110[p3] (Neve and Kurnit, 1983). First, an EcoRI/HindIII fragment (positions 1-1075 in CAATP1) (Brandt et al., 1988) was ligated into the EcoRI and HindIII sites of the homologous recombination vector Xα7N. The resulting ligation reaction was treated with PstI to reduce background transformants and then was used to transform E. coli MC1061[p3]. The ampicillin- and tetracycline-resistant transformants containing the inserted sequence were selected by in situ colony hybridization using the 32P-labeled EcoRI/HindIII fragment as a probe (Maniatis et al., 1982).

One million bacteriophage from a human genomic library (Law et al., 1978) were used to infect E. coli MC1061[p3] cells containing the Xα7N-CAATP1 recombinant plasmid. After overnight growth, bacteriophage were eluted from the plate by incubation in suspension medium (50 mm Tris, 10 mm MgSO4, 0.2% gelatin, pH 7.5) at room temperature for 4 h. The eluted bacteriophage were titered on E. coli MC1061[p3]. The ampicillin-, tetracycline-, and chloramphenicol-resistant transformants containing the inserted sequence were selected by in situ colony hybridization using the 32P-labeled EcoRI/HindIII fragment as a probe (Maniatis et al., 1982).

Restriction map of the insert of one of the recombinant bacteriophage isolated by homologous recombination (Xβ6686) was generated (data not shown). Based on this map, a 5773-bp PstI/SphI fragment that was in the intron between exons B and C was used to probe a different human genomic library. One million bacteriophage from this library were screened by in situ plaque hybridization, and the positive plaques were carried to clonal purity by successive rescreening as described above for the screening of bacteriophage containing cDNA inserts.

DNA from bacteriophage containing genomic inserts was examined by Southern blot analysis. The DNA was digested with EcoRI, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane (Hybond-N). The blot was hybridized with the 32P-labeled EcoRI/HindIII fragment as a probe (Maniatis et al., 1983). Free nucleotides were separated from labeled DNA by chromatography over Sephadex G-25. Oligonucleotide probes were labeled with [γ-32P]ATP and T4 polynucleotide kinase (Greene and Wettstein, 1987), and free label was removed by two precipitations with ammonium acetate and ethanol in the presence of 2 μg of carrier RNA.

RESULTS

PMCA1 mRNA Distribution—The mRNAs derived from the PMCA1 gene have four known spliced forms designated PMCA1a–PMCA1d (Shull and Greeb, 1988; Strehler et al., 1989). The four mRNAs arise from the insertion of various exons at a common point between exons A and C. The inserted exons are designated B', B', and B'; and their combinations that contribute to the different PMCA1 mRNAs are shown in Fig. 1. A Southern transfer of the PCR products was probed with oligonucleotides specific to the intron-exon junctions of PMCA1 that should occur within the amplified region. Fig. 3A shows the autoradiograph of a transfer that was probed with a PMCA1 exon C-specific probe. This probe hybridizes to all four of the PMCA1 spliced forms. PMCA1a was found in spinal cord, brain, skeletal muscle, and heart. PMCA1b was in all tissues examined. PMCA1c was found in skeletal muscle, heart, spinal cord, and brain. PMCA1d was found only in heart and skeletal muscle. The results presented in Figs. 3 and 4 (A, C, and D) are typical of the results obtained from four independent experiments. Fig. 4B is a typical result from three independent experiments.

To validate the data obtained with the PMCA1 exon C-specific probe, the transfer was hybridized with probes that were specific for each of the PMCA1 variable exons. In Fig. 3B, the transfer was probed with an oligonucleotide specific to PMCA1 exon B. This probe should have detected PMCA1a, PMCA1c, and PMCA1d-derived products, and it is seen that bands corresponding to the expected sizes of these products hybridized with the probe. This is also seen in Fig. 3C, where an exon B'-specific probe hybridized with PMCA1a- and PMCA1d-derived products, and in Fig. 3D, where an exon B''-specific probe detected PMCA1a-derived products. To further verify the product thought to be derived from PMCA1a, the membrane was hybridized with an oligonucleotide that would only hybridize with the exon B'-'exon C junction. The results of this hybridization are shown in Fig. 3E. The PMCA1a-derived material was found in brain and spinal cord as expected. Products corresponding to PMCA1a could also be seen in skeletal muscle and heart in a longer exposure of this autoradiograph (data not shown). Several faint bands around 510 bp were detected with the exon B-, B', B'', and C-specific probes, but not with the exon B''-exon C junction probe (Fig. 3). These may be due to as yet uncharacterized PMCA1 isoform mRNAs that have exons inserted between exons B'' and C.

PMCA2 mRNA Distribution—Fig. 4A shows the same transfer used in Fig. 3 reprobed with a PMCA2 exon C-specific probe. The expected amplification product derived from the known PMCA2 mRNA was found in brain, liver, spinal cord, and adrenal gland. In spinal cord, there were bands at ~344 and 390 bp that also hybridized with the PMCA2 exon C3-specific probe. These bands may correspond to other spliced forms of PMCA2.
PMCA3 mRNA Distribution—The amplification products derived from the PMCA3 mRNA were resolved on a separate gel and probed with a PMCA3 exon C-specific probe. Fig. 4B shows the result of this hybridization. Based on the cDNA sequence, the PMCA3 mRNA would have a 154-bp exon between exons A and C, resulting in a 358-bp amplification product. However, by homology to other Ca\(^{2+}\)-ATPase cDNAs, Greb and Shull (1989) predicted another mRNA that would lack this exon and designated it PMCA3b, whereas the original isolate was designated PMCA3a. The amplification product generated from the PMCA3b mRNA would be 204 bp in length. PMCA3a-derived material was seen in spinal cord and brain. Products derived from the putative PMCA3b mRNA were present in adrenal gland, spinal cord, and brain. In addition to amplification products derived from PMCA3a and PMCA3b, three other bands were observed primarily in spinal cord, which may be additional alternately spliced versions of PMCA3.

PMCA4 mRNA Distribution—The Southern blot used to generate the data in Figs. 3 (A-E) and 4A was reprobed with an oligonucleotide specific for PMCA4 exon C. The autoradiograph resulting from this hybridization is shown in Fig. 4C. Amplification products derived from the PMCA4a mRNA (Strehler et al., 1990) were present in all tissues. Also, an additional band at 342 bp was detected primarily in skeletal muscle, small intestine, heart, spinal cord, and brain. This band was found to be derived from a new spliced form of PMCA4, (PMCA4b), reported here. The sequence of hPMCA4b was determined (see below), the same transfer used in Fig. 4C was hybridized with a probe specific for PMCA4 exon B (PMCA4b-specific). The results, shown in Fig. 4D, verify that the band seen at 342 bp with the exon C-specific probe was derived from the new isoform (PMCA4b).

Characterization of Bovine and Human PMCA4 cDNA Clones—In our initial studies (Brandt et al., 1988), screening of a λgt11 bovine brain cDNA expression library with rabbit anti-human erythrocyte Ca\(^{2+}\)-ATPase antibodies resulted in the isolation of a 1.5-kilobase pair cDNA (designated CAATP1). This cDNA represented the 3'-terminus of what is now known as the PMCA4a mRNA. Our subsequent cloning strategy was to use this 3'-end cDNA sequence to obtain adjacent sequences by primer extension of the mRNA. This strategy was adopted to avoid the potential problem of generating a chimeric structure based on the isolation of cDNA fragments derived from other Ca\(^{2+}\)-ATPase isoform mRNAs with similar sequences. These experiments resulted in the isolation of a partial cDNA for an alternately spliced form of PMCA4 (here designated PMCA4b).

The screening of 260,000 bacteriophage from a bovine brain cDNA library constructed using Oligo A (Fig. 2) as a primer yielded six positive plaques. The recombinant phage containing the largest insert was designated λBB-4. This insert terminated at a naturally occurring EcoRI site at position 942 (Fig. 2 and contained in the sequence shown in Fig. 5). A second bovine brain cDNA library was constructed using an oligonucleotide complementary to sequences in the insert of λBB-4 as a primer for cDNA synthesis. The screening of 300,000 bacteriophage from this library resulted in the isolation of 28 positive clones, one of which (ABB-6) contained an insert that yielded the rest of the sequence shown in Fig. 5.

The strategy employed in sequencing the inserts of ABB-4 and λBB-6 is presented schematically in Fig. 2. The DNA of
FIG. 4. Southern blots of PCR amplification products derived from hPMCA2–hPMCA4 mRNAs. A, the Southern blot used in Fig. 3 was probed with a probe specific for exon C of rPMCA2. B, a Southern blot prepared as described for Fig. 3, but with hPMCA3-specific PCR products made using the PMCA3-specific primers described under "Experimental Procedures." The blot was probed with a probe specific for exon C of hPMCA3. C, the blot used in Fig. 3 was probed with a probe specific for exon C of hPMCA4. D, the blot used in Fig. 3 was probed with a probe specific for exon B of hPMCA4. Markers were HindIII-digested pBR322 DNA.

FIG. 5. Partial cDNA sequence of Ca2+-ATPase isoform (bPMCA4b) from bovine brain and its deduced protein sequence. This is the combined DNA sequence of the cDNA inserts from λBB-6 (positions 1–1356) and λBB-4 (positions 943–1497) isolated from bovine brain cDNA libraries. In keeping with the nomenclature proposed by Greeb and Shull (1988), the combined sequences of these bacteriophage λ cDNA inserts are designated bPMCA4b (see Footnote 2). Only the sequence of BB-4 up to the second EcoRI site is presented because the sequence downstream of that point has previously been reported as CAATPl (Brandt et al., 1988). The nucleotides are numbered with the 5' end of the sequence, and the amino acids are numbered to the right of the sequence.

the insert from AB-6 that was sequenced only in one direction (nucleotides 1–942 in Fig. 5) was confirmed by comparison with the human homolog of this sequence described below. When differences between bovine and human sequences occurred, the autoradiograph from the bovine cDNA sequencing reactions was examined for ambiguous nucleotide assignments. In all cases, it was possible to assign the nucleotide in the bovine sequence unambiguously. The combined sequences of the inserts from λBB-6 and λBB-4 up to the EcoRI site that marks the beginning of CAATPl are shown in Fig. 5.

Because of the availability of many more high quality human brain cDNA libraries than bovine brain libraries, we elected to screen one of these libraries to isolate full-length cDNA clones, rather than to continue in the bovine system.
Comparison of bovine PMCA4b with the homologous region of human PMCA4a. The cDNA sequence of new Ca\(^{2+}\)-ATPase isoform (hPMCA4b) and comparison of its deduced protein sequence to hPMCA4a. This represents a part of the sequence of the cDNA insert of lHB-4 isolated from a human fetal brain cDNA library. The numbering of the DNA sequence is as described by Strehler et al. (1990) for hPMCA4a and for hPMCA4b up to the point of exon insertion. Asterisks designate stop codons. The caret indicates the Thr residue phosphorylated by protein kinase C in PMCA4a (Wang et al., 1991).

The screening of 300,000 recombinant bacteriophage from a human fetal brain cDNA library with the upstream EcoRI fragment from the insert of lBB-4 resulted in the isolation of 18 positive plaques. Examination of the sequence of the insert from one of these isolates (lHB-4) showed that the sequence was identical to hPMCA4 (Strehler et al., 1990) from residues 2176 to 3403, except that a 178-bp stretch was inserted immediately after the sequences encoding the calmodulin-binding domain at position 3309. The sequence of what is presumed to be a new exon is shown in Fig. 6 with a portion of flanking sequence from the PMCA4a sequence presented by Strehler et al. (1990). To be consistent with the nomenclature used by Strehler et al. (1989) and Greeb and Shull (1989), we presumably a new exon is shown in Fig. 6 with a portion of the C terminus of the PMCA4a-encoded protein. The caret indicates the Thr residue phosphorylated by protein kinase C in PMCA4a (Wang et al., 1991).

Comparison of bovine PMCA4b with the homologous region of human PMCA4a showed that the deduced protein sequences were nearly identical (Fig. 7). In general, only conservative substitutions were observed where there were differences between the sequences. The only other difference found was the insertion of a single alanine residue in the bovine sequence at position 458. This high degree of conservation across species is also seen between rat and human PMCA1 and rat and human PMCA2.

Characterization of Exon ABC-containing Region of PMCA4 Gene—To verify the existence of the new exon B suggested by the sequence of the PMCA4b cDNA, we probed a human genomic DNA library with an EcoRI/HindIII fragment from CAATP1 by homologous recombination techniques and chose six isolates for examination. Southern blot analysis with probes from two different regions of PMCA4 showed that a single 4.3-kilobase pair PstI-generated fragment in an isolate designated \(\lambda\)H6 contained the probe sequence as well as upstream sequences (data not shown). A smaller PstI/SphI fragment of the 4.3-kilobase pair PstI-generated fragment that contained only DNA from the intron between exons B and C was used to probe a human genomic DNA library by filter hybridization. Eight positive plaques were selected and taken to clonal purity, and their DNA was subjected to Southern analysis. Probing of the Southern blot with oligonucleotide probes specific to each of the exons in the variable region of PMCA4b (exons A–C) showed that one isolate (\(\lambda\)H6\(_{5}\)) contained all three exons and that each exon was on a separate EcoRI fragment (data not shown). The EcoRI fragments that hybridized with the exon-specific probes were subcloned into the plasmid vector pGEM7Zf (+) for sequence analysis.

The sequences determined for the intron-exon junctions of the exons that constitute the variably spliced region of PMCA4 are shown in Fig. 8. All of the region that constitutes exon B was sequenced in the genomic DNA and was found to contain no additional introns. The sequences upstream of exons B and C have the requisite structures that distinguish intron sequences at the acceptor site of a splice junction. The 2 intronic residues immediately adjacent to the exons are AG, and sequences upstream from that point are very pyrimidine-rich (Breathnach and Chambon, 1981). Also, several sequences that could serve as internal splice recognition sequences (Keller and Noon, 1984), where "lariat" intermediates are formed, were found upstream of each exon (underlined sequences).

The sequences downstream of exons A and B contained sequences that would distinguish them as intron donor sites. The consensus sequence for intron donor sites is GT(A/G)AGT (Breathnach and Chambon, 1981). The residues immediately downstream of exon A were GTACCT. The obligate GT residues at the 5'-end of the intron were present, but two of the next four nucleotides deviated from the consensus sequence. The sequence of the intron immediately adjacent to the 3'-end of exon B was GTGAGT. This sequence is an exact match for the intron donor site consensus sequence.

DISCUSSION

The recent cloning of several cDNAs corresponding to the Ca\(^{2+}\)-ATPases has indicated that there is great diversity in the mRNAs that encode different forms of the enzyme. It should have been possible to use exon-specific oligonucleotide probes in standard Northern blot analysis to determine the
tissue-specific distribution of the various Ca\textsuperscript{2+}-ATPase mRNAs. However, we have found by several different methods that these mRNAs appear to be very low abundance messages; and their detection, even with high specificity activity cRNA or DNA probes, has been extremely difficult (data not shown). Even though the methodology we employed for Northern blotting was nearly identical to that used by others to examine the PMCA mRNAs, our results have been more comparable to those reported by Strehler et al. (1990) than to those of Greeb and Shull (1989) or, more recently, Kuo et al. (1991). We have determined that the integrity of the RNA on our Northern blots is intact by hybridization with other probes (e.g., amyloid precursor protein cDNA). Therefore, it is difficult to assess the reason for the discrepancies between our observations and other reports. Based on these results, we judged that hybridization of Northern blots with end-labeled oligonucleotide probes would not be feasible for determining the tissue-specific distribution of the various Ca\textsuperscript{2+}-ATPase isoforms. Therefore, we have used the combination of PCR and Southern blotting to examine the distribution of various isoform mRNAs in tissues. The results presented in this report further extend the range of this mRNA diversity by demonstrating that the tissue distribution of each of the alternately spliced Ca\textsuperscript{2+}-ATPase mRNAs is unique and that other spliced forms may exist.

The tissue distribution of PMCA4 mRNAs has not been previously reported. The results in Fig. 4 (C and D) show that there are at least two forms of the PMCA4 mRNA that are alternately spliced in the region encoding the regulatory domain of the Ca\textsuperscript{2+}-ATPase. One of these PMCA4 variants has been previously published (Strehler et al., 1990) and is designated PMCA4a. The characterization of another PMCA4 isoform mRNA (PMCA4b) and its gene is reported herein. PMCA4a is found in all tissues examined, and its tissue distribution is identical to that of PMCA1b. The PMCA4b mRNA was found primarily in the extracellular tissues skeletal muscle, small intestine, heart, spinal cord, and brain. The ability of this technique to detect the previously unknown PMCA4b mRNA demonstrates its utility in examining mRNA expression in complex systems such as the Ca\textsuperscript{2+}-ATPase mRNA families. Comparison of the deduced protein sequences of bovine and human PMCA4b shows a high degree of similarity. The fact that there is more homology between the same isoform in different species than among the different isoforms in the same species suggests that the Ca\textsuperscript{2+}-ATPase genes diverged and their protein products developed specialized functions early in mammalian evolution. This high degree of conservation across species also suggests that there is strong selective pressure to maintain the primary structure of the various Ca\textsuperscript{2+}-ATPase isoforms and that, by extension, the tissue-specific splicing of the mRNAs encoding these sequences would also be maintained.

The tissue-specific expression of the known Ca\textsuperscript{2+}-ATPase mRNAs is summarized in Table I. These results show that the expression of the Ca\textsuperscript{2+}-ATPase mRNAs exhibits a great deal of diversity. Comparison of the data presented here to those of Greeb and Shull (1988) from adult rat tissues suggests that there are also development- or species-specific differences in mRNA expression. In the case of PMCA1, the isoform seen by Greeb and Shull (1988) in all tissues was PMCA1b. The methodology they used could not distinguish among the alternately spliced forms of the PMCA1 mRNAs, so PMCA1b masked any differences that might have been seen in the other PMCA1 mRNAs. These authors found the PMCA2 mRNA in adult rat brain and liver, which agrees with the data presented here for human fetal tissues. However, we detected no PMCA2 mRNA in human fetal kidney, whereas Greeb and Shull found it in adult rat kidney. PMCA3 mRNAs had previously been found primarily in brain and skeletal muscle, with minor amounts in tissues of the digestive tract of adult rat. PMCA3a and PMCA3b mRNAs also were expressed in human fetal brain, but no PMCA3 mRNAs were detected in human fetal skeletal muscle or small intestine.

With regard to developmental regulation of mRNA expression, it is clear that the expression of different PMCA1--PMCA3 mRNAs changes from embryonic to adult brain. This may also occur in other tissues and could explain our inability to detect the PMCA2 mRNA in fetal human kidney or the PMCA3 mRNA in human fetal skeletal muscle since these genes may not be expressed until adulthood in these tissues. The use of different Ca\textsuperscript{2+}-ATPases to perform the same function in human and rat cannot be precluded. For example, human kidney might have evolved using the PMCA1b or PMCA4a Ca\textsuperscript{2+}-ATPase, whereas rat kidney may have evolved the use of the PMCA2 Ca\textsuperscript{2+}-ATPase for the same function.

During the course of the PMCA mRNA distribution analysis, we also isolated a cDNA encoding an alternately spliced form of PMCA4 (PMCA4b). The existence of the PMCA4b mRNA was predicted by the PCR-based detection method discussed above. PMCA4b differs from PMCA4a by the addition of a single exon inserted between the exons homologous to exons A and C of PMCA1. The inserted exon from human PMCA4 is 178 bp in size, which is ~30 bp longer than the combined exons B, B', and B'' of PMCA1, to which it has some homology. Despite this homology, the data presented here show no evidence for mRNA processing that would lead to PMCA4 mRNAs equivalent to PMCA1c and PMCA1d.
even though the minimum required sequences for exon acceptor sites (Breathnach and Chambon, 1981) are present in PMCA4 exon B in the regions homologous to the PMCA1 acceptor sites.

We have previously proposed that the carboxyl terminus of PMCA4b is a calmodulin homolog that binds to the calmodulin-binding site and inhibits enzyme activity until it is displaced by interaction with Ca²⁺-calmodulin (Brandt et al., 1988). This proposal is in agreement with studies of the human erythrocyte Ca²⁺-ATPase that showed that enzymatic activity, but PMCAla does not behave 3’ to that of PMCA4b changes the reading frame. The data presented here that these isoforms are ex-

PMCA1 also exhibits a similar phenomenon wherein isoforms of the PMCA4 class of Ca²⁺-ATPases that may have lost splicing in this region of the coding sequence. This further supports the proposal that the use of alternate splicing to change the mRNA sequences encoding the regulatory domains is common in the differential expression of Ca²⁺-ATPase isoforms. There are mRNAs for at least nine different iso-

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### Table II

| mRNA | Spleen | Thymus | Pancreas | Skeletal muscle | Kidney | Liver | Lung | Small intestine | Heart | Adrenal gland | Spinal cord | Brain |
|------|--------|--------|---------|-------------|--------|------|------|----------------|-------|--------------|------------|-------|
| PMCA1a | +      | +      | +       | +           | +      | +    | +    | +              | +     | +            | +          | +     |
| PMCA1b | +      | +      | +       | +           | +      | +    | +    | +              | +     | +            | +          | +     |
| PMCA1c | +      | +      | +       | +           | +      | +    | +    | +              | +     | +            | +          | +     |
| PMCA1d | +      | +      | +       | +           | +      | +    | +    | +              | +     | +            | +          | +     |
| PMCA2  | +      | +      | +       | +           | +      | +    | +    | +              | +     | +            | +          | +     |
| PMCA3a | +      | +      | +       | +           | +      | +    | +    | +              | +     | +            | +          | +     |
| PMCA3b | +      | +      | +       | +           | +      | +    | +    | +              | +     | +            | +          | +     |
| PMCA4a | +      | +      | +       | +           | +      | +    | +    | +              | +     | +            | +          | +     |
| PMCA4b | +      | +      | +       | +           | +      | +    | +    | +              | +     | +            | +          | +     |
Analysis of Ca\textsuperscript{2+}-ATPase Regulatory Domain mRNA Splicing

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