Molecular Cloning of a Third Isoform of the Calmodulin-sensitive Plasma Membrane Ca\(^{2+}\)-Transporting ATPase That Is Expressed Predominantly in Brain and Skeletal Muscle*

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A complementary DNA for a third isoform of the calmodulin-sensitive plasma membrane Ca\(^{2+}\) pump has been isolated from a rat brain cDNA library. The nucleotide sequence of the 5.1-kilobase pair cDNA has been determined, and the amino acid sequence of the protein, designated PMCA3, has been deduced. PMCA3 is 1159 amino acids in length and has an \(M_r\) of 127,300. It exhibits 81% and 85% amino acid identity, respectively, to isoforms 1 and 2 (PMCA1 and PMCA2) of the plasma membrane Ca-ATPase. The transcript encoding PMCA3 is similar to that of PMCA1 in that it contains a sequence in the 3′-untranslated region that has the potential to encode an alternative calmodulin binding domain and carboxyl terminus. The tissue distribution of mRNAs encoding isoforms 1, 2, and 3 has been determined by Northern blot hybridization analyses. PMCA1 mRNAs are expressed in all tissues examined, suggesting that this protein may serve as a housekeeping form of the enzyme. However, PMCA2 and PMCA3 mRNAs exhibit a high degree of tissue specificity. PMCA2 mRNAs are expressed predominantly in brain and heart, whereas PMCA3 mRNAs are expressed predominantly in brain and skeletal muscle.

Because of its role as a second messenger, the concentration of cytoplasmic Ca\(^{2+}\) must be regulated with great precision. The calmodulin-sensitive plasma membrane Ca\(^{2+}\) pump provides much of the capability for the fine-tuning of intracellular Ca\(^{2+}\) levels and, in order to achieve the necessary degree of control, the activity of the enzyme is highly regulated (1, 2). As intracellular Ca\(^{2+}\) concentrations rise above a threshold level, formation of a Ca\(^{2+}\)/calmodulin complex occurs which then binds to and activates the enzyme (2). Subsequent lowering of intracellular free [Ca\(^{2+}\)] leads to dissociation of calmodulin and inactivation of the enzyme. The enzyme is also regulated by other means. An acidic protein inhibitor has been identified in brain (3) and in erythrocytes (4). In cardiac (5) and skeletal muscle (6), the plasma membrane Ca-ATPase is regulated by cAMP-dependent protein kinase and that of vascular smooth muscle is regulated indirectly by cGMP-dependent protein kinase (7–9).

It has recently become apparent that the calmodulin-sensitive plasma membrane Ca-ATPase is not a single enzyme but, rather, consists of a family of enzymes encoded by multiple genes. Thus, tissue-specific differences in the regulation of the Ca\(^{2+}\) pump may be due in part to tissue-specific expression of different isoforms of the enzyme. The structure of two distinct isoforms from rat brain have been determined by molecular cloning procedures (10). PMCA1 is 1,176 amino acids in length with a \(M_r\) of 129,500 and PMCA2 is 1,198 amino acids in length with a \(M_r\) of 132,600. The two proteins exhibited 82% amino acid identity to each other and are encoded by separate genes. Analysis of the nucleotide sequence of the cDNA encoding isoform 1 suggested the possibility that alternative splicing of the primary transcript might yield mRNAs encoding PMCA1 variants that differ in their regulatory properties. A cDNA encoding one of the proposed PMCA1 variants has been isolated from a human teratoma library and shown to encode a protein of 1,220 amino acids with a \(M_r\) of 134,700 (11). It contains an alternative calmodulin binding domain and a cAMP-dependent phosphorylation site that might also be encoded by an alternatively spliced exon since a rat brain cDNA has been identified (10) which lacks a 33-nucleotide sequence capable of encoding this site.

It is clear that there are isoforms of the plasma membrane Ca-ATPase in addition to those that have already been characterized. The amino acid sequences of a large number of peptides from the human erythrocyte plasma membrane Ca-ATPase have been determined (11), and since they exhibit only 86% and 84% identity to the corresponding regions of isoforms 1 and 2, respectively, the erythrocyte protein must be a different isoform. In addition, a partial cDNA for a bovine brain plasma membrane Ca\(^{2+}\) pump has been characterized (12) that encodes a carboxyl-terminal sequence that differs substantially from those of isoforms 1 and 2.

To identify cDNAs for additional isoforms of the plasma membrane Ca-ATPase, we screened a rat brain library at reduced stringency with a restriction fragment of an isoform 1 cDNA. A cDNA was identified that encodes the complete

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05807.

1The nomenclature used for the plasma membrane Ca-ATPase (PMCA) isoforms was introduced in Ref. 10. The numbering of isoforms PMCA1, PMCA2 (10), and PMCA3 is according to the order in which these cDNAs were identified. Each protein seems to be encoded by a separate gene. It is now established that there are at least two regulatory variants of PMCA1 that arise as a result of alternative splicing (see Refs. 10 and 11). PMCA1a is used to designate the 1176-amino acid variant (10), and PMCA1b is used to designate the 1220-amino acid variant (11). PMCA3a refers to the 1159-amino acid protein encoded by the cDNA shown in Fig. 2, and PMCA3b refers to a hypothetical 1206-amino acid variant that would be encoded by the cDNA if a 154-nucleotide sequence (residues 3301–3454) were removed by an alternative splicing event (see text).
sequence of a third distinct isofrom of the plasma membrane Ca\(^{2+}\) pump. Amino acid similarity comparisons indicated that PMCA3 does not correspond to the erythrocyte enzyme or the enzyme encoded by the bovine brain cDNA (12). The data presented here and in previous studies (10–12) suggest that there is a high degree of diversity among plasma membrane Ca-ATPases due to the existence of multiple genes and alternative processing of the primary transcripts.

**EXPERIMENTAL PROCEDURES**

**Rat Brain cDNA Library**—The library used in this study consisted of 40,000 colonies with inserts of 3.3–5.5 kb that were tailed with deoxyctosine and annealed to tailed-g pBR322 plasmid vector. The library was an independent plating of the same cDNA fraction used in the isolation of cDNAs for isoforms 1 and 2 of the plasma membrane Ca-ATPase (10). The procedures used to synthesize the cDNA, plating of the library, and preparation of replica filters were described in previous studies (13, 14).

**Isolation of cDNAs**—Replica filters were prehybridized overnight at 61 °C in 6 × SSC, 5 × Denhardt's solution, 0.1% SDS, and 100 μg of denatured salmon sperm DNA/ml (see Ref. 15 for composition of SSC and Denhardt's solution). Hybridization was performed at 61 °C for 44 h in the same solution containing 1 × 10\(^{-6}\) cpn/ml of a 32P-labeled 1.45-kb AaII restriction fragment from a PMCA1 cDNA (nucleotides 2408-3660 of clone RB 11-1 described in Ref. 10). The probe was labeled with [\(^{32}\)P]dCTP to a specific activity of 1 × 10\(^{8}\) cpn/ml using the random primer Oligolabelling Kit from Pharmacia LKB Biotechnology Inc. The filters were washed two times for 5 min each at room temperature in 3 × SSC, 0.1% SDS and then two times for 45 min each at 61 °C in 3 × SSC, 0.1% SDS. The filters were then washed for 20 min at 65 °C in 0.1% SDS and then hybridized with a 1.45-kb AaII fragment that was used to screen the library. Hybridization and washing conditions were the same as those used in the screening the library. Following autoradiography, the cDNAs were classified based on their hybridization patterns. Two cDNAs that exhibited hybridization patterns differing from those of the PMCA1 and PMCA2 cDNAs were examined by restriction endonuclease mapping. The nucleotide sequence of one of these cDNAs was determined using the chemical cleavage procedure of Maxam and Gilbert (16). 100% of the cDNAs were examined by autoradiography, and 10 cDNAs that gave positive hybridization signals were colony-purified.

**Analysis of cDNAs**—In order to group them into classes, the cDNAs were examined by Southern blot hybridization. Plasmid DNA was prepared by the alkaline lysis procedure (15), digested with the restriction endonuclease Sau3A, and fractionated by electrophoresis in 1% agarose. cDNAs encoding PMCA1 and PMCA2 were treated in the same manner and run in parallel lanes. The DNA was transferred to a Nylon membrane and hybridized with the 32P-labeled 1.45-kb AaII fragment that was used to screen the library. Hybridization and washing conditions were the same as those used in screening the library. Following autoradiography, the cDNAs were classified based on their hybridization patterns. Two cDNAs that exhibited hybridization patterns differing from those of the PMCA1 and PMCA2 cDNAs were examined by restriction endonuclease mapping. The nucleotide sequence of one of these cDNAs was determined using the chemical cleavage procedure of Maxam and Gilbert (16). 100% of the cDNAs were examined by autoradiography, and 10 cDNAs that gave positive hybridization signals were colony-purified. To determine whether any of the cDNAs might encode isoforms other than isoforms 1, 2, and 3 (described below), the cDNAs were washed as described in the high stringency protocol except that the 60 °C wash in 0.1 × SSC, 0.5% SDS was omitted.

Restriction fragments used as isoform-specific hybridization probes were taken from the 3' and 5' ends of cDNAs encoding PMCA1, PMCA2, and PMCA3. The PMCA1 and PMCA2 cDNAs are described in Ref. 10; the PMCA3 cDNA is described in this study. Each of the 3' probes was from the untranslated sequence. Each of the 5' probes included untranslated sequence and part of the 5' coding sequence that is not highly conserved between isoforms. These probes are: i) PMCA1, clone RB 11-1, Rfl-ScaI fragment, nucleotides 29-383, ii) PMCA1, clone RB 11-2, Hpal-PstI fragment, nucleotides 4977-4777 (this clone contains 539 base pairs of 3' untranslated sequence that extend beyond that reported for clone RB 11-1 in Ref. 10 and 144 base pairs that extend beyond that occurring in the human PMCA1 cDNA reported in Ref. 11); iii) PMCA2, clone RB L6, Apal-Ncol fragment, nucleotides 295-311; iv) PMCA2, clone RB 14-4, ScaI-NciI fragment, nucleotides 5790-6580; iv) PMCA3, clone RB 7-2, SchI-ScaI fragment, nucleotides 474-115; v) PMCA3, clone RB 7-2, ClaI-SpeI fragment, nucleotides 2937-4238.

**RESULTS AND DISCUSSION**

**Isolation of Plasma Membrane Ca-ATPase cDNAs**—Based on the results of several studies discussed in the introduction (10–12), it is apparent that there are additional plasma membrane Ca-ATPase isoforms other than isoforms 1, 2, and 3 (Fig. 8) consisted of a mixture of the following restriction fragments: i) PMCA1, clone RB 11-1, HindIII-BglII fragment, nucleotides 2545-2962 encoding amino acids 761-900; ii) PMCA2, clone RB L6, BglII-PvuII fragment, nucleotides 2859-3111; iii) PMCA2, clone RB L6, SchI-ScaI fragment, nucleotides 5790-6580; iv) PMCA3, clone RB 7-2, SchI-ScaI fragment, nucleotides 474-115; v) PMCA3, clone RB 7-2, ClaI-SpeI fragment, nucleotides 2937-4238.

**The CDNA probe used to detect mRNAs that might correspond to plasma membrane Ca-ATPase isoforms other than isoforms 1, 2, and 3 (Fig. 8) consisted of a mixture of the following restriction fragments: i) PMCA1, clone RB 11-1, HindIII-BglII fragment, nucleotides 2545-2962 encoding amino acids 761-900; ii) PMCA2, clone RB L6, BglII-PvuII fragment, nucleotides 2859-3111; iii) PMCA2, clone RB L6, SchI-ScaI fragment, nucleotides 5790-6580; iv) PMCA3, clone RB 7-2, HindIII-NdeI fragment, nucleotides 2937-2962 encoding amino acids 761-900.**

**RESULTS AND DISCUSSION**

The data described in this study show that there are at least three distinct Ca-ATPase isoforms in rat brain. Each of the three isoforms is encoded by a separate gene, and the open reading frames of the three cDNAs do not overlap. The amino acid sequence of the third Ca-ATPase isoform is significantly different from that of the other two isoforms. The cloning of these cDNAs provides a basis for further study of the functional properties of these isoforms.

**REFERENCES**

1. The abbreviations used are: kb, kilobases; SDS, sodium dodecyl sulfaite; FITC, fluorescein isothiocyanate; F348A, 5'β-fluorescein-5'-fluorpyrination; CAF, γ-[N-2-chloroethyl-N-methylamine], benzylamide ATP.
endonuclease Sau3A, which cleaves DNA at a 4-base pair recognition sequence, and examined by Southern blot hybridization analysis. Since recognition sites for this enzyme occur frequently, the resulting hybridization patterns provide a fingerprint of each cDNA.

Three cDNAs gave hybridization patterns that were characteristic of PMCA1, four gave patterns that were characteristic of PMCA2, and one cDNA gave a hybridization pattern that differed slightly from that of PMCA1. Nucleotide sequence analysis of the latter cDNA demonstrated that it was derived from an alternatively spliced transcript of the PMCA1 gene.2 The protein it encodes is the same as the PMCA1 variant cloned from a human teratoma cDNA library (11). The two remaining cDNAs gave hybridization patterns that were substantially different from those of the PMCA1 and PMCA2 cDNAs (data not shown). Detailed restriction endonuclease mapping was performed, and the nucleotide sequence

2 T. Keeton and G. Shull, unpublished observations.
of the longer of the two unique cDNAs, RB 7-2, was determined.

Clone RB 7-2 Encodes a Third Isoform of the Calmodulin-sensitive Plasma Membrane Ca-ATPase—The restriction map and sequencing strategy used in the analysis of clone RB 7-2 is shown in Fig. 1, and the nucleotide and deduced amino acid sequences are shown in Fig. 2. The cDNA is 5094 nucleotides in length and encodes a protein of 1159 amino acids that has a M, of 127,300. The apparent translation start codon is in a strong context for initiation of translation (20) and is preceded by an in-frame stop codon at nucleotides -39 to -37. The 695-nucleotide 5'-untranslated sequence contains a 117-codon open reading frame (nucleotides -371 to -21) that begins with an ATG triplet and is in a poor context for translation initiation. This reading frame also contains two codon open reading frame (nucleotides -371 to -21) that is in length and encodes a protein of 1159 amino acids that has a strong context for initiation of translation (20) and is preceded by an in-frame stop codon at nucleotides -39 to -37.

This 5'-untranslated sequence contains an additional 23 nucleotides that include four repeats of the sequence AAAG. Therefore, it seems likely that the 3' end of RB 7-2 corresponds to an internal A-rich region in the 3'-untranslated sequence of the corresponding mRNA. This probably occurred during cDNA synthesis as a result of hybridization of the oligo(dT) primer to this A-rich region.

Northern blot analysis, described below, suggests that the mRNA from which RB 7-2 was derived contains an additional 2 kb of untranslated sequence.

The protein encoded by RB 7-2 is clearly an isoform of the calmodulin-sensitive plasma membrane Ca-ATPase. A sequence near the carboxyl terminus exhibits a 16/17 match with the corresponding region of the calmodulin-sensitive plasma membrane Ca-ATPase (21). The first 19 amino acids of this sequence exhibits a 16/17 match with the corresponding region of the calmodulin-sensitive plasma membrane Ca-ATPase (21). The first 19 amino acids of this sequence (residues 1082-1100), which has the potential to form an amphipathic α-helix, is identical with the corresponding sequence of the protein encoded by RB 7-2. The protein encoded by RB 7-2 is clearly an isoform of the calmodulin-sensitive plasma membrane Ca-ATPase. A sequence near the carboxyl terminus exhibits a 16/17 match with the corresponding region of the calmodulin-sensitive plasma membrane Ca-ATPase (21). The first 19 amino acids of this sequence (residues 1082-1100), which has the potential to form an amphipathic α-helix, is identical with the corresponding sequence of the protein encoded by RB 7-2.
untranslated sequences of RB 7-2 are very different from those of the PMCA1 and PMCA2 cDNAs. From these data, we conclude that RB 7-2 was derived from a transcript of a third PMCA gene.

It should be noted that PMCA3 is not the same enzyme as the erythrocyte Ca\textsuperscript{2+} pump. Sequences of peptides from the human erythrocyte Ca-ATPase, which include 184 residues, have been determined by direct amino acid sequencing (11). These sequences exhibit only 81% identity to the corresponding sequences of PMCA3. The amino acid mismatches seem too extensive to represent species differences in the same isoform. It seems likely that the erythrocyte enzyme is encoded by a different gene than that of PMCA1 and PMCA2.

Comparison of the Amino Acid Sequence of PMCA3 with Those of PMCA1 and PMCA2—A comparison of the deduced amino acid sequences of three plasma membrane Ca-ATPases is shown in Fig. 3. The general organization of PMCA3 is the same as that of PMCA1 and PMCA2. The amino terminus of each primary translation product begins with the same 4-amino acid sequence, and the three proteins can be aligned throughout their lengths with the introduction of only a few gaps. PMCA3 exhibits 81% identity to PMCA1 and 85% identity to PMCA2. Major hydrophobic regions that may be transmembrane domains show a higher degree of amino acid similarity than the overall average. The calmodulin binding domain and carboxyl terminus of PMCA3 shown in Fig. 3 exhibit greater similarity to PMCA1 than to PMCA2. Below, we discuss the alternative carboxyl-terminal region that is known to occur in a variant form of PMCA1 and show that a similar variant form of PMCA3 might also occur. These alternative carboxyl-terminal regions are similar to that of PMCA2.

As there is evidence that the cardiac (5) and skeletal muscle (6) plasma membrane Ca-ATPases are regulated by cAMP-dependent protein kinases, we examined the sequence of each isoform for potential phosphorylation sites. All three isoforms contain the sequence, Arg-Lys-Ser-Met-Ser (amino acids 566-570 in PMCA3), which is located near the lysine residue found in the FITC site. Ser-570 is a potential cAMP-dependent phosphorylation site (24). This sequence is also present in the fast-twitch and slow-twitch sarcoplastic reticulum Ca-ATPases (25) and in a closely related enzyme that may be an endoplasmic reticulum Ca\textsuperscript{2+} pump (29). The occurrence of this sequence in both intracellular and plasma membrane Ca-ATPases encoded by six different genes suggests that it may have functional significance. Other residues that might conceivably serve as cAMP-dependent phosphorylation sites are Ser-1112 and Ser-1122 of PMCA3 and Ser-1140 of PMCA1. These sites occur within or very near the calmodulin binding site.

Alternative Splicing of the Primary Transcript May Yield PMCA3 Isoforms with Different Regulatory Domains—Examination of a PMCA1 cDNA in a previous study provided suggestive evidence that alternative splicing of the primary transcript might yield additional isoforms that differ in their calmodulin binding domains and in their potential for regulation by cAMP-dependent kinases (10). This was based on the observation that the 3'-untranslated region of the PMCA1 cDNA contains a sequence with the potential to encode an alternative calmodulin binding site B domain and a carboxyl terminus that is similar to that of PMCA2. Excision of a 154-nucleotide sequence immediately following codon 1117 would result in a mRNA encoding the alternative calmodulin binding domain and carboxyl terminus. A cDNA corresponding to this variant isoform was isolated from a human teratoma library (11), and we have identified a rat brain cDNA that also encodes this isoform. In this discussion, we refer to the alternative isoform, which is 1220 amino acids in length, as PMCA1b and the original isoform, which is 1176 amino acids in length, as PMCA1a.

Sequence comparisons have also indicated the possibility of a third PMCA1 variant. One of the PMCA1 cDNAs identified earlier contains an interesting 33-nucleotide sequence within the region encoding the alternative carboxyl terminus (10). The position of this sequence, which encodes a potential cAMP-dependent phosphorylation site (11), corresponds to the location of an inhibitory domain that has been identified.
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**FIG. 5. Northern blot analysis of PMCA1 mRNAs.** Poly(A)+ RNA from the indicated tissues was analyzed by Northern blot hybridization using probes from the 3'-untranslated region and the 5' end of a PMCA1 cDNA as described under "Experimental Procedures." The probe and autoradiographic exposure times were: A, 3' probe, 5.5 h; B, 3' probe, 16 h; C, 3' probe, 70 h; D, 5' probe, 16 h; E, 5' probe, 48 h. The positions and sizes (in kb) of the RNA markers are shown on the right.

An examination of the sequence of clone RB 7-2 suggests that the PMCA3 gene may have the potential to encode a variant isoform with a different calmodulin binding site and carboxyl terminus. In this respect, the organization of clone RB 7-2 is identical with that of the PMCAl cDNA described earlier (10). If a 154-nucleotide sequence of the PMCA3 mRNA (nucleotides 3301-3454) were inserted into the corresponding position of the PMCA2 cDNA, then a termination codon would occur in the same location as in the PMCA1a and PMCA3a transcripts. The results of Northern blot analyses of PMCA2 mRNAs, described below, are consistent with the possibility of alternatively spliced PMCA2 transcripts. It is also of interest that the sequences of several peptides from the carboxyl-terminal region of the human erythrocyte plasma membrane Ca2+ pump (11, 21) resemble amino acid sequences in the carboxyl-terminal regions of PMCA1b, PMCA2, and the hypothetical PMCA3b (Fig. 4) rather than those of PMCA1a and PMCA3a.

**Northern Blot Analyses of mRNAs Encoding PMCA1, PMCA2, and PMCA3**—To determine the tissue distribution...
The autoradiographic exposure time was 22 h. The position and sizes probe, 21 h; of poly(A)+ RNA from a variety of rat tissues. Isoform-specific isoforms 1, stringency using a probe consisting of poly(A)+ RNA from a variety of rat tissues. Isoform-specific isoforms, Northern blot hybridization analysis was performed at moderate stringency using a mixture of cDNA probes encoding a highly conserved region of isoforms 1, 2, and 3 of the plasma membrane Ca2+ pump, we performed Northern blot analyses of poly(A)+ RNA from a variety of rat tissues. Isoform-specific cDNA probes were prepared from the 3' untranslated regions and from sequences that included 5'-untranslated and 5'-coding regions. The coding sequences included in the 5' probes were not highly conserved among the three isoforms, and no evidence of cross-hybridization was observed. PMCA1 transcripts were expressed in all tissues examined (Fig. 5), suggesting that the PMCA1 isoform might function as the major "housekeeping" plasma membrane Ca-ATPase. The highest mRNA levels were observed in brain, lung, and intestine; the lowest levels were observed in pancreas, testes, and liver. Major mRNAs of approximately 5.5-6.0 kb and 7.6-7.8 kb were observed with both the 3' and 5' probes, which gave the same hybridization patterns. A minor mRNA of 4.8 kb was seen in most tissues after long exposures. The basis for the different mRNA sizes has not been determined. The PMCA1 cDNAs that have been characterized (10, 11) are under 5 kb in length and do not contain polyadenylation signals. Evidently, they lack a considerable amount of untranslated sequence, and it is possible that the missing sequence contains several polyadenylation signals that might account for the different mRNA sizes. A second possibility is that the 5.5-6.0-kb and 7.6-7.8-kb mRNAs correspond to alternatively spliced transcripts of the PMCA1 gene. If this is the case, then there may be alternative splicing patterns other than those involving the 154- and 33-nucleotide sequences since removal of either one or both of these regions would change the mRNA size by less than 200 nucleotides. There seem to be some minor variations in mRNA sizes in different tissues (for example, compare the upper band in small intestine and large intestine) that might be due to alternative splicing involving these regions but this is uncertain.

Expression of PMCA2 transcripts exhibited a high degree of tissue specificity (Fig. 6). The 3' probe detected a major 7.6-kb mRNA and a minor 5.8-kb mRNA in brain. The same probe detected a 7.9-kb mRNA in heart. Faint bands were also present in mRNA from several other tissues. The 7.6-kb brain transcript probably corresponds to the 7-kb PMCA2 cDNA isolated in a previous study (10), and the 7.9-kb heart mRNA could correspond to an alternatively spliced transcript analogous to that of PMCA1a. The 5' probe identified the same mRNAs detected by the 3' probe but also identified several smaller mRNAs. In brain it identified two additional transcripts of 6.5 and 5.4 kb, and in heart it identified transcripts of 6.5, 5.7, and 4.7 kb. Using the 5' probe, faint bands were observed in a number of other tissues. For example, the 7.6- and 6.5-kb mRNAs are present at low levels in uterus and in kidney; liver contains the 7.6- and 5.7-kb mRNAs.

PMCA3 expression also exhibited a high degree of tissue specificity (Fig. 7). The 3' probe hybridized with a major 7.3-7.7-kb mRNA in brain and in skeletal muscle and detected a mRNA of the same size at low levels in testes. The 5' probe detected the same mRNAs but also detected a major mRNA of 4.3-4.6 kb in skeletal muscle, a minor 5.8-kb mRNA in skeletal muscle, and a minor 4.2-kb mRNA in brain. At long exposures, faint bands were detected at approximately 7.5 kb in stomach, small intestine, and large intestine.

Evidence for a Fourth Isoform of the Plasma Membrane Ca-ATPase That Is Expressed Predominantly in Uterus and Stomach—As discussed above, the erythrocyte plasma membrane Ca-ATPase seems to be an isoform different from PMCA1, -2, or -3, and the enzyme encoded by a partial bovine brain cDNA (12) also seems to be a different isoform. To search for evidence of mRNAs that might encode additional isoforms, Northern blot hybridization analysis was performed at moderate stringency using a mixture of cDNA probes encoding a highly conserved region of isoforms 1, 2, and 3
(Fig. 8). A mRNA of approximately 8.8 kb was detected in uter us and in stomach that does not correspond to the mRNAs identified in Figs. 5-7. This suggests that a fourth isof orm of the plasma membrane Ca-ATPase is expressed in these tissues.

Potential for Additional Diversity due to Multiple Genes and Alternative Processing of Primary Transcripts—The cloning and characterization of cDNAs for two forms of PMCA1 and one form of PMCA2 (10, 11) demonstrated that the genetic basis for diversity among the calmodulin-sensitive plasma membrane Ca-ATPases is due both to the existence of multiple genes and to alternative splicing of the primary transcripts. Cloning of the PMCA3 cDNA described in this study provides direct evidence for the existence of a third plasma membrane Ca-ATPase gene, and it seems clear from an examination of the available amino acid sequence data (11) for the erythrocyte Ca<sup>2+</sup> pump that it is encoded by a fourth gene. Evidence for additional genes besides those encoding PMCA1, -2, and -3 is also provided by the cloning of a partial bovine brain cDNA (12) and by the Northern blot data, in which an 8.8-kb mRNA was identified that did not correspond to mRNAs for PMCA1, -2, or -3 (Fig. 8). Thus, there appear to be at least four genes and possibly more.

Sequence comparisons demonstrate the potential for alternatively processed PMCA3 transcripts similar to those encoding PMCA1a and PMCA1b. As the carboxyl-terminal regions of PMCA2 and the erythrocyte plasma membrane Ca<sup>2+</sup> pump are similar to those of PMCA1b and the hypothetical PMCA3b, it seems possible that there may be alternatively spliced transcripts that encode the "a" forms of these enzymes. The 33-nucleotide sequence encoding amino acids 1172-1182 of PMCA1b, which includes a potential cAMP-dependent phosphorylation site, might also be alternatively spliced (10). This amino acid sequence is in a location corresponding to an inhibitory domain of the erythrocyte Ca<sup>2+</sup> pump (28). Although the corresponding regions of PMCA2 and PMCA3b do not include consensus sequences for phosphorylation by cAMP-dependent kinases, it is conceivable that they serve some other regulatory function and, therefore, the possibility of alternative splicing of the nucleotide sequence encoding this region should be considered.

For the PMCA2 (Fig. 6) and PMCA3 (Fig. 7) mRNAs, a more complex hybridization pattern was observed when the blots were hybridized with probes from the 5' end of the cDNA than that observed with the 3'-untranslated sequence probes. By analogy to the PMCA1 transcripts, mRNAs encoding putative "a" and "b" forms of PMCA2 and PMCA3 would be expected to hybridize with the 3' probes. The 7.9-kb PMCA2 transcript in heart and the 7.6-kb PMCA2 transcript in brain, that are recognized by the 3' probe, could correspond to the a and b transcripts, respectively. A number of additional bands were observed when the blots were hybridized with the 5' PMCA2 ad PMCA3 probes, and it is possible that these correspond to other alternatively spliced mRNAs. If this is the case, then diversity among the plasma membrane Ca-ATPases might be much greater than previously suspected.

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