Molecular Cloning and Tissue Distribution of Alternatively Spliced mRNAs Encoding Possible Mammalian Homologues of the Yeast Secretory Pathway Calcium Pump[†,⊖]

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ABSTRACT: Rat stomach and testis cDNAs corresponding to two alternatively spliced mRNAs encoding variants of a P-type ion-transport ATPase that closely resembles the yeast secretory pathway Ca^{2+} pump have been isolated and characterized. A partial kidney cDNA was identified previously using an oligonucleotide probe corresponding to part of the sarcoplasmic reticulum Ca^{2+}-ATPase [Gunteski-Hamblin, A., Greeb, J., & Shull, G. E. (1988) *J. Biol. Chem. 263*, 15032–15040]. In the present study, we first isolated and characterized a stomach cDNA that contains the entire coding sequence. The 919 amino acid enzyme has the same apparent transmembrane organization and contains all of the conserved domains present in other P-type ATPases. Northern blot analyses demonstrate that 3.9- and 5-kilobase mRNAs corresponding to the cDNA were present in all tissues examined, suggesting that the protein it encodes performs a housekeeping function. Rat testis also contained a 3.7-kilobase mRNA that hybridized with a probe from the 5′ end of the stomach cDNA but did not hybridize with a probe from the 3′ end. Cloning and characterization of cDNAs corresponding to the smaller testis mRNA revealed that it is derived from the same gene but encodes a variant of the enzyme in which the C-terminal residue, Val-919, is replaced by the sequence Phe-919-Tyr-Pro-Lys-Ile-923. Similarity comparisons show that the two enzymes are more closely related to the known Ca^{2+} pumps than to other P-type ATPases. They exhibit 23% amino acid identity with the plasma membrane Ca^{2+}-ATPase, 33% identity with the sarcoplasmic reticulum Ca^{2+}-ATPase, and 50% identity with the yeast secretory pathway Ca^{2+}-ATPase. On the basis of these comparisons, it seems likely that they are mammalian homologues of the yeast secretory pathway Ca^{2+} pump.

ATP-dependent Ca^{2+} pumps of the P-type family of enzymes play an essential role in the control of intracellular Ca^{2+} concentrations (Pietrobon et al., 1990). Two distinct classes of mammalian P-type Ca^{2+} pumps have been identified and extensively characterized using biochemical and molecular biological techniques. These are the plasma membrane Ca^{2+}-ATPases (PMCA class), which extrude Ca^{2+} from the cell, and the sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCA class), which sequester Ca^{2+} within intracellular storage vesicles. Full-length cDNAs encoding four calmodulin-sensitive plasma membrane Ca^{2+} pumps (Shull & Greeb, 1988; Verma et al., 1988; Greeb & Shull, 1989; Strehler et al., 1990) and three sarco(endo)plasmic reticulum Ca^{2+} pumps (MacLennan et al., 1985; Brandl et al., 1986; Burk et al., 1989) that are the products of separate genes have been identified, and additional diversity is generated by alternative splicing of exons encoding the C-terminal regions (Strehler et al., 1989; Brandl et al., 1987; Lytton & MacLennan, 1988; Gunteski-Hamblin et al., 1988). By pumping Ca^{2+} out of the cytoplasm, both classes of pumps serve to maintain low resting levels of cytosolic Ca^{2+}, and SERCA pumps have the additional function of maintaining intracellular Ca^{2+} stores that must be available for release into the cytosol during Ca^{2+} signaling events.

There is evidence for the existence of additional mammalian Ca^{2+} pumps besides those that have already been characterized. ATP-dependent Ca^{2+} uptake activity has been shown in coated vesicles (Blitz et al., 1977), lysosomes (Klempner, 1985), nuclei (Nicotera et al., 1989), and Golgi vesicles of lactating mammary glands (Virk et al., 1985; Watters, 1984; Neville et al., 1981). The coated vesicle Ca^{2+} pump reacts with antibodies directed against the SR Ca^{2+}-ATPase (Blitz et al., 1977) and, therefore, could be a member of the SERCA class of pumps, but its exact identity has not been determined. The Ca^{2+} pump identified in nuclei requires calmodulin, like the PMCA pumps, but has a relatively low sensitivity to vanadate (Nicotera et al., 1989). There is insufficient information regarding the biochemical properties of the nuclear membrane pump or of the lysosomal pump to determine whether they might be related to the SERCA or PMCA pumps. The Golgi Ca^{2+} pump is sensitive to vanadate (Virk et al., 1985), a characteristic of the P-type ATPases, but has other characteristics, such as a different apparent Ca^{2+} affinity and insensitivity to quercetin, that seem to distinguish it from the PMCA and SERCA pumps (Virk et al., 1985).

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[⊖] The nucleotide sequences in this paper have been submitted to GenBank under Accession Numbers M93017 (Figure 2) and M93018 (Figure 6).

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[1] Abbreviations: SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SDS, sodium dodecyl sulfate; FITC, fluorescein isothiocyanate; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; ClRATP, γ-4-[*N*-(2-chloroethyl)-*N*-methylamino]benzylamide ATP; kb, kilobases; nts, nucleotides; bp, base pairs.

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In a previous study (Gunteski-Hamblin et al., 1988), directed at the isolation and characterization of cDNAs encoding Ca^{2+}-ATPases of intracellular and plasma membranes, we identified a partial rat kidney cDNA, clone RK9-11, encoding a protein that exhibited significant amino acid similarity to several known Ca^{2+} pumps; however, on the basis of the degree of similarity it did not appear to be a member of the SERCA or PMCA families of Ca^{2+}-ATPases. This raised the possibility that it represented a third distinct class of mammalian Ca^{2+} pump. In the present study, we have isolated and characterized cDNAs derived from two alternatively spliced mRNAs that encode variants of the protein which differ in their C-terminal sequences. Amino acid similarity comparisons show that the two enzymes are closely related to a yeast P-type ATPase (Rudolph et al., 1989) that seems to function as a secretory pathway Ca^{2+} pump.

EXPERIMENTAL PROCEDURES

Rat Brain, Kidney, Stomach, and Testis cDNA Libraries. The rat brain, kidney, and stomach cDNA libraries used in this study, each consisting of 50 000 colonies, were the same libraries used previously in the isolation of various transport. ATPase cDNAs (Gunteski-Hamblin et al., 1988; Shull et al., 1986; Shull & Lingrel, 1986). The rat testis library, obtained from Stratagene, was prepared using cDNA of greater than 2.5 kb in length and the phage vector Uni-ZAP XR. A total of 70 000 plaques were plated on 26 master plates. Replica filters were prepared and processed basically as described by Maniatis et al. (1982).

Isolation and Characterization of cDNAs. Replica filters from brain, kidney, and stomach cDNA libraries were prehybridized overnight at 58 °C in 6× SET, 1× Denhardt's solution, 0.1% SDS, and 100 μg of denatured salmon sperm DNA/mL [see Maniatis et al. (1982) for the composition of SET and Denhardt's solution) and then hybridized in the same solution for 36 h at 58 °C with a 519-bp *Sty*I–*Nsi*I fragment (corresponding to nts 2245–2764 shown in Figure 2) of clone RK9-11 (Gunteski-Hamblin et al., 1988) that had been labeled with [α-^{32}P]dCTP using the random primer oligolabeling kit from Pharmacia LKB Biotechnology, Inc. Filters were washed three times for 30 min each at 58 °C in 6× SET, 0.1% SDS, and analyzed by autoradiography. cDNAs that gave positive hybridization signals colony purified, and plasmid DNA was isolated by the alkaline lysis procedure (Maniatis et al., 1982). Restriction mapping and hybridization analysis indicated that all of the clones belonged to the same class as clone RK9-11 and identified clone RS10-31 as the longest cDNA. DNA sequence analysis of clone RS10-31 was performed by the chemical cleavage procedure (Maxam & Gilbert, 1980).

The rat testis library was screened using two *Pst*I restriction fragments of clone RS10-31 (nts 776–1965 and nts 1966–2518) under essentially the same conditions as described above. Ten cDNAs that gave positive hybridization signals were plaque purified and the pBluescript phagemid portion of the λ Uni-ZAP vector, which contained the cDNA insert, was excised and used to infect XL1 Blue cells as described by the supplier (Stratagene). Restriction mapping and hybridization analysis demonstrated that the testis cDNAs belonged to two closely related classes. DNA sequence analysis was performed by the dideoxy chain termination method (Sanger et al., 1977) using the Pharmacia T7 sequencing kit (Pharmacia LKB Biotechnology, Inc.) and the Sequenase Version 2.0 Kit (U.S. Biochemical). Computer analysis of the DNA sequence was performed using the program of Wernke and Thompson (1989) (DNANALYZE, version 5.1).

Northern Blot Hybridization Analysis. Poly(A)$^+$ RNA was isolated from adult CD rats, and Northern blots were prepared exactly as described previously (Burk et al., 1989). Five micrograms of poly(A)$^+$ RNA, which had been denatured with glyoxal, was run in each lane. Labeling of probes with [α-^{32}P]dCTP, hybridization at 42 °C in a solution containing 50% formamide, and washing of filters were also performed exactly as described (Burk et al., 1989). Restriction fragments used as hybridization probes were the following: (i) *Bst*BI–*Nsi*I fragment (nts 2278–2764) from the C-terminal coding region of clone RS10-31; (ii) *Cla*I–*Hpa*I fragment (nts 2987–3595) from the 3′ untranslated sequence of clone RS10-31; (iii) *Bgl*I–*Bsp*HI fragment (nts 3861–4376) from the 3′ untranslated sequence of testis clone RT7-1; and (iv) *Bsu*36I–*Dra*I fragment (nts 2841–3315 plus 52 nts that seem to have been artifactually derived from an unrelated mRNA) from the 3′ untranslated sequence of clone RT5-1, which was derived from the testis-specific mRNA.

RESULTS

Isolation and Sequence Analysis of cDNAs Encoding a New Member of the P-Type Family of Ion Transport ATPases. In one of our earlier studies, we screened rat brain, kidney, and stomach cDNA libraries using an oligonucleotide probe based on a 23 amino acid sequence from the ATP binding site of the SR Ca^{2+}-ATPase (Gunteski-Hamblin et al., 1988). Among the cDNAs identified was a kidney cDNA, clone RK9-11, that encoded part of a P-type ATPase that exhibited similarity to both the sarcoplasmic reticulum and plasma membrane Ca^{2+} pumps. To isolate a full-length cDNA encoding this pump, we rescreened the original brain, kidney, and stomach libraries using a cDNA probe from clone RK9-11. One brain, three stomach, and two kidney cDNAs (including clone RK9-11) were identified. The cDNAs were colony purified and examined by restriction endonuclease mapping and Southern blot hybridization analysis. On the basis of the hybridization patterns, it was apparent that all of the cDNAs were derived from a single class of mRNA. Four of the five new clones isolated did not contain the complete open reading frame, but the remaining cDNA, stomach clone RS10-31, had an insert of 3.8 kb, and subsequent sequence analysis showed that it contained the entire coding sequence.

In a later phase of this study, we identified testis cDNAs corresponding to both an alternatively spliced testis-specific mRNA that contained a different 3′ end and an mRNA that was apparently identical to the form cloned from the stomach, brain, and kidney libraries. One of the testis cDNAs for the latter mRNA, clone RT7-1, encoded the same protein as the brain, kidney, and stomach cDNAs but contained additional 3′ untranslated sequences that were missing from stomach cDNA RS10-31. The restriction maps for clones RS10-31 and RT7-1 are shown in the upper part of Figure 1, and the nucleotide and deduced amino acid sequences are shown in Figure 2. The bottom part of Figure 1 shows the restriction maps for the cDNAs derived from the testis-specific mRNA, which contains alternative C-terminal coding and 3′ untranslated sequences. The Northern blot analysis used to identify the testis-specific mRNA and the cloning of testis cDNAs for both classes of mRNA will be described in detail in a later section.

The composite cDNA sequence for clones RS10-31 and RT7-1 is 4645 nts in length and has a 2757-nt open reading frame. The ATG triplet beginning at nucleotide +1 is the probable translation initiation site since it is in an acceptable context for initiation of translation (Kozak, 1987) and is

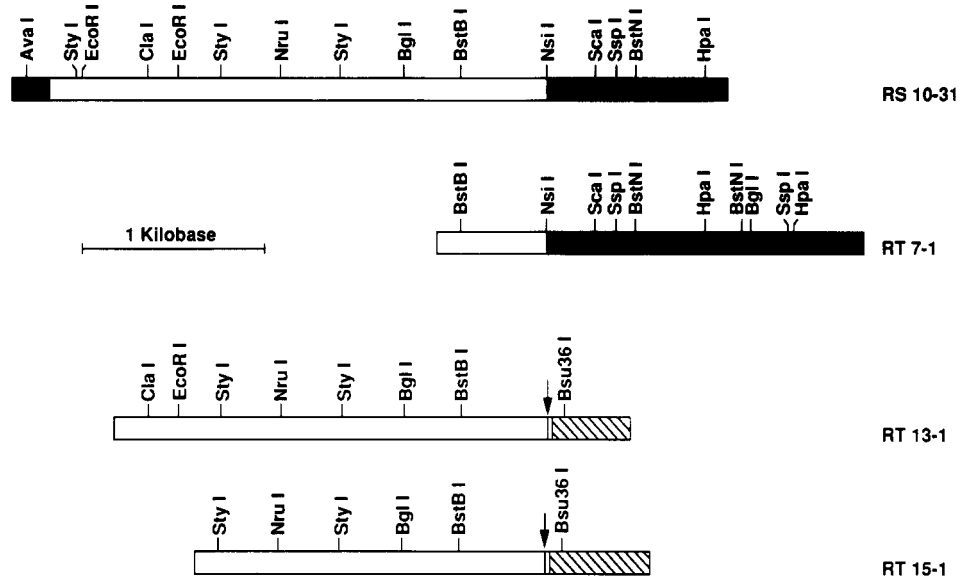

FIGURE 1: Restriction map for rat stomach and testis cDNAs representing alternatively spliced mRNAs encoding variants of a novel P-type ATPase. Stomach cDNA RS10-31 (nts −178 to 3655) and testis cDNA RT7-1 (nts 2139–4467) are derived from an mRNA expressed in all tissues. Testis cDNAs RT13-1 (nts 407–3174) and RT15-1 (nts 838–3315) correspond to the testis-specific mRNA with alternative C-terminal coding and 3′ untranslated sequences. The cDNAs shown here were used to compile the composite sequences shown in Figures 2 and 6. Open areas represent the coding regions, and dark or hatched areas represent untranslated regions. The arrow indicates the point at which the sequence of the testis-specific transcript diverges from that of the transcript expressed in all tissues. The direction and extent of sequencing are indicated by arrows (lower arrows, coding strand; upper arrows, noncoding strand).

preceded by an in-frame stop codon which begins at nt −32. The 178-nt 5′ untranslated sequence contains two other ATG triplets, which generate small open reading frames of 10 and 12 codons before encountering in-frame stop codons. One of these small open reading frames (nts −7 to +29) overlaps the 919-codon open reading frame. Because the ATG triplet for this 12-codon open reading frame is in a very poor context for initiation of translation, it seems unlikely that it would interfere with initiation from the ATG triplet at nt +1. However, if it did interfere, then initiation would most likely occur at position +49, thereby eliminating the first 16 amino acids of the predicted translation product. The 1690-nt 3′ untranslated sequence terminated with a poly(A) tract that began 19 nts following a consensus polyadenylation signal (Proudfoot & Brownlee, 1976). Two additional potential polyadenylation signals, at nts 3173–3178 and nts 3994–3999, are present in the 3′ untranslated sequence. Northern blot analysis, discussed below, suggests that the first site is utilized in the production of a 3.9-kb mRNA whereas the site at nts 4423–4429 is used for a 5-kb mRNA.

The deduced primary translation product is 919 amino acids in length, has an M_r of 100 500, and contains all of the conserved domains commonly found in P-type ion-transport ATPases. The apparent phosphorylation site (Post & Kume, 1973), Asp-350, occurs in the sequence Cys-Ser-Asp-Lys-Thr-Gly-Thr, which has been found in all eukaryotic P-type ATPases that have been characterized so far. The enzyme also contains sequences resembling those that bind FITC in the Na,K-ATPase (Farley et al., 1984), SR Ca-ATPase (Mitchinson et al., 1982), and plasma membrane Ca-ATPase (Filoteo et al., 1987), as well as sequences resembling the binding site for the ATP analogues FSBA (Ohta et al., 1986) and ClRATP (Ovchinnikov et al., 1987) in the Na,K-ATPase (see Figure 8). The regions that bind FITC, FSBA, and ClRATP are thought to form part of the ATP binding site.

Hydropathy analysis (Figure 3) indicates that the protein contains up to 10 potential membrane-spanning domains and has a transmembrane organization that is identical to that of other P-type transport ATPases (Shull & Greeb, 1988). The enzyme has four major hydrophobic domains in the N-terminal half of the protein that correspond to known transmembrane domains in the other ATPases. The C-terminal half of the enzyme contains six hydrophobic domains corresponding to regions that have been proposed as possible membrane-spanning domains in other transport ATPases. Because the RS10-31 protein has the same apparent transmembrane organization and contains all of the conserved domains characteristic of the mammalian P-type ATPases, it is clear that is represents a new mammalian member of this family of enzymes.

Northern Blot Hybridization Analyses. To determine the mRNA tissue distribution, poly(A)$^+$ RNAs from 12 rat tissues were analyzed by Northern blot hybridization using probes from the coding sequence and the 3′ untranslated sequence of clone RS10-31 (Figure 4). Both probes detected mRNAs of approximately 3.9 kb and 5 kb in all tissues examined except testis. As described below, the two probes gave different patterns in testis, suggesting the existence of an alternatively spliced mRNA. An additional hybridization signal corresponding to an mRNA of 6 kb was also seen in some tissues. The levels of expression of the 3.9- and 5-kb mRNAs were slightly higher in brain, lung, and stomach than in other tissues but, in general, the mRNA concentrations were remarkably uniform in the various tissues, suggesting that the enzyme performs a housekeeping function.

It should be noted that the 3′ untranslated sequence probe, consisting of nts 2987–3595, spans the potential polyadenylation signal observed at nts 3173–3178. Because the relative intensity of the hybridization signal for the 5-kb mRNA, compared with that of the 3.9-kb mRNA, was greater for the 3′ untranslated sequence probe than for the coding sequence probe (compare panels B and C of Figure 4), it is likely that this polyadenylation signal is used for the 3.9-kb mRNA. Consistent with this conclusion, we found that a probe consisting of nts 3861–4376 hybridized only with the 5- and 6-kb mRNAs (Figure 5). The Northern blot data gave no indication that the potential polyadenylation signal at nts 3994–

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                                                                                                                    TCCCTGCCACCATCCTGCTCGTTCCCTC  -151
CGGACGCGGCTGGGCTCCGTGTGGCGCCACCGTGCCGCCAGCAGCGCCCGTCGCCCGAGGCGCGTGGGGCGCCCCCGCAGCCCCGCGATGTGGCCGTGGCTGACACTAAAGACTTTGTAGCCATCAACCAGAGTGCAGTTTCGATGGAAA  -1

ATGAAGGTTGCACGATTTCAAAAGATCCCTAATGTTGAAAATGAGACAATGATCCCTGTACTGACATCCAAGAGAGCAAGTGAGTTAGCAGTCAGTGAGGTCGCAGGCCTTCTCCAGGCTGATCTTCAGAATGGCTTAAACAAATCTGAA  150
MetLysValAlaArgPheGlnLysIleProAsnValGluAsnGluThrMetIleProValLeuThrSerLysArgAlaSerGluLeuAlaValSerGluValAlaGlyLeuGluGlnAlaAspLeuGlnAsnGlyLeuAsnLysSerGlu
                    10                  20                  30                  40                  50
GTTAGTCATAGGCGAGCCTTCCATGGCTGGAATGAGTTTGATATCAGTGAAGATGAACCATTATGGAAGAAGTACATTTCTCAGTTTAAAAATCCCCTCATCATGCTGCTTCTGGCTTCGGCAGTCATCAGCGTTTTAATGCGTCAGTTT  300
ValSerHisArgArgAlaPheHisGlyTrpAsnGluPheAspIleSerGluAspGluProLeuTrpLysLysTyrIleSerGlnPheLysAsnProLeuIleMetLeuLeuLeuAlaSerAlaValIleSerValLeuMetArgGlnPhe
                    60                  70                  80                  90                  100
GATGATGCCGTCAGTATCACTGTGGCAATACTGATTGTTGTCACCGTGGCCTTTGTTCAGGAATATCGTTCAGAAAAATCTTTAGAAGAATTAAGTAAACTTGTGCCACCAGAATGCCATTGTGTTCGTGAAGGAAAATTGGAGCATACA  450
AspAspAlaValSerIleThrValAlaIleLeuIleValValThrValAlaPheValGlnGluTyrArgSerGluLysSerLeuGluGluLeuSerLysLeuValProProGluCysHisCysValArgGluGlyLysLeuGluHisThr
                    110                 120                 130                 140                 150
CTTGCCCGAGACTTGGTTCCAGGTGATACAGTTTGTCTGTCTGTGGGGGACAGAGTTCCTGCTGACTTACGCTTATTCGAGGCCGTGGATCTTTCCATCGATGAGTCTAGCTTGACAGGAGAGACAACTCCTTGTTCTAAGGTGACCGCT  600
LeuAlaArgAspLeuValProGlyAspThrValCysLeuSerValGlyAspArgValProAlaAspLeuArgLeuPheGluAlaValAspLeuSerIleAspGluSerSerLeuThrGlyGluThrThrProCysSerLysValThrAla
                    160                 170                 180                 190                 200
CCTCAACCAGCTGCTACCAATGGGGATCTTGCATCAAGAAGTAACATTGCCTTCATGGGGACTCTGGTCAGATGTGGCAAAGCAAAGGGTATTGTCATTGGAACAGGAGAGAATTCTGAATTTGGAGAGGTCTTTAAGATGATGCAGGCA  750
ProGlnProAlaAlaThrAsnGlyAspLeuAlaSerArgSerAsnIleAlaPheMetGlyThrLeuValArgCysGlyLysAlaLysGlyIleValIleGlyThrGlyGluAsnSerGluPheGlyGluValPheLysMetMetGlnAla
                    210                 220                 230                 240                 250
GAAGAAGCACCAAAAACGCCTCTGCAGAAGAGCATGGACCTCTTGGGCAAGCAGCTGTCCTTTTACTCCTTTGGTATAATAGGTATCATCATGTTGGTTGGCTGGTTACTAGGGAAAGACATTCTGGAAATGTTCACTATTAGTGTAAGT  900
GluGluAlaProLysThrProLeuGlnLysSerMetAspLeuLeuGlyLysGlnLeuSerPheTyrSerPheGlyIleIleGlyIleIleMetLeuValGlyTrpLeuLeuGlyLysAspIleLeuGluMetPheThrIleSerValSer
                    260                 270                 280                 290                 300
TTGGCTGTAGCTGCAATTCCTGAAGGTCTGCCTATTGTGGTCACAGTGACACTAGCCCTTGGTGTTATGAGAATGGTGAAGAAAAGGGCTATTGTAAAGAAATTGCCTATTGTTGAAACGCTGGGCTGCTGTAATGTGATTTGTTCAGAT  1050
LeuAlaValAlaAlaIleProGluGlyLeuProIleValValThrValThrLeuAlaLeuGlyValMetArgMetValLysLysArgAlaIleValLysLysLeuProIleValGluThrLeuGlyCysCysAsnValIleCysSerAsp
                    310                 320                 330                 340                 •
AAAACTGGAACCCTGACGAAGAATGAGATGACTGTTACTCACATCCTCACTTCAGACGGCCTGCATGCTGAGGTTACTGGAGTTGGCTACAATCAGTTTGGTGAAGTGATTGTTGATGGTGATGTTGTTCATGGATTCTATAACCCAGCT  1200
LysThrGlyThrLeuThrLysAsnGluMetThrValThrHisIleLeuThrSerAspGlyLeuHisAlaGluValThrGlyValGlyTyrAsnGlnPheGlyGluValIleValAspGlyAspValValHisGlyPheTyrAsnProAla
                    360                 370                 380                 390                 400
GTTAGCAGAATTGTTGAGGCGGGCTGTGTGTGCAACGATGCTGTCATTAGGAACAACACTCTGATGGGAAAGCCAACTGAAGGAGCCTTAATCGCTCTCGCGATGAAGATGGGTCTTGATGGACTGCAACAAGACTATATCAGAAAAGCT  1350
ValSerArgIleValGluAlaGlyCysValCysAsnAspAlaValIleArgAsnAsnThrLeuMetGlyLysProThrGluGlyAlaLeuIleAlaLeuAlaMetLysMetGlyLeuAspGlyLeuGlnAspTyrIleArgLysAla
                    410                 420                 430                 440                 450
GAATACCCTTTTAGTTCTGAGCAGAAATGGATGGCTGTTAAGTGTGTGCACCGAACACAGCAGGACAGACCAGAGATTTGTTTTATGAAGGGTGCTTATGAGCAGGTGATTAAGTATTGTACTACGTACAACAGCAAAGGGCAGACTTTG  1500
GluTyrProPheSerSerGluGlnLysTrpMetAlaValLysCysValHisArgThrGlnGlnAspArgProGluIleCysPheMetLysGlyAlaTyrGluGlnValIleLysTyrCysThrThrTyrAsnSerLysGlyGlnThrLeu
                    460                 470                 480                 490                 500
GCACTTACCCAGCAGCAGAGAGATTTGTACCAACAAGAGAAGGCCCAGATGGGCTCGGCAGGACTCCGAGTTCTTGCACTGGCGTCTGGTCCGGACCTGGGGCAGCTGACCCTCCTTGGCCTGGTAGGAATCATTGACCCTCCGAGAACT  1650
AlaLeuThrGlnGlnGlnArgAspLeuTyrGlnGlnGluLysAlaGlnMetGlySerAlaGlyLeuArgValLeuAlaLeuAlaSerGlyProAspLeuGlyGlnLeuThrLeuLeuGlyLeuValGlyIleIleAspProProArgThr
                    510                 520                 530                 540                 550
GGTGTGAAAGAAGCCGTCACAACACTCATTGCCTCAGGAGTCTCCATCAAAATGATCACAGGAGACTCTCAGGAGACTGCAATTGCAATCGCTAGTCGTCTGGGATTGTATTCTAAAACTTCACAGTCTGTGTCTGGAGAAGAAGTAGAT  1800
GlyValLysGluAlaValThrThrLeuIleAlaSerGlyValSerIleLysMetIleThrGlyAspSerGlnGluThrAlaIleAlaIleAlaSerArgLeuGlyLeuTyrSerLysThrSerGlnSerValSerGlyGluGluValAsp
                    560                 570                 580                 590                 600
ACAATGGAAGTCCAGCACCTTTCACAGATAGTGCCAAAGGTTGCAGTATTTTACAGAGCAAGCCCAAGACACAAGATGAAAATTATCAAGTCTCTACAAAAGAACGGGTCAGTTGTAGCCATGACAGGAGATGGGGTAAATGATGCAGTG  1950
ThrMetGluValGlnHisLeuSerGlnIleValProLysValAlaValPheTyrArgAlaSerProArgHisLysMetLysIleIleLysSerLeuGlnLysAsnGlySerValValAlaMetThrGlyAspGlyValAsnAspAlaVal
                    610                 620                 630                 640                 650
GCTCTGAAGGCTGCAGATATTGGAGTTGCAATGGGCCAGACTGGCACAGATGTTTGCAAAGAGGCTGCGGACATGATCTTGGTGGATGATGATTTCCAAACTATAATGTCTGCAATAGAAGAGGGTAAAGGCATTTATAATAACATTAAA  2100
AlaLeuLysAlaAlaAspIleGlyValAlaMetGlyGlnThrGlyThrAspValCysLysGluAlaAlaAspMetIleLeuValAspAspAspPheGlnThrIleMetSerAlaIleGluGluGlyLysGlyIleTyrAsnAsnIleLys
                    660                 670                 680                 690                 700
AATTTTGTTAGATTTCAACTGAGCACGAGTATAGCAGCATTAACTTTAATCTCATTGGCTACGTTAATGAACTTTCCTAACCCTCTCAATGCAATGCAGATTTTGTGGATCAATATTATAATGGATGGACCCCCAGCTCAGAGCCTTGGA  2250
AsnPheValArgPheGlnLeuSerThrSerIleAlaAlaLeuThrLeuIleSerLeuAlaThrLeuMetAsnPheProAsnProLeuAsnAlaMetGlnIleLeuTrpIleAsnIleIleMetAspGlyProProAlaGlnSerLeuGly
                    710                 720                 730                 740                 750
GTAGAGCCAGTGGATAAAGATGTCATTCGAAAACCCCCTCGGAACTGGAAGGACAGCATTTTGACAAAAAACTTGATCCTTAAAATACTTGTTTCATCAATAATCATTGTCTGTGGGACTTTGTTTGTCTTCTGGCGAGAGCTTCGAGAC  2400
ValGluProValAspLysAspValIleArgLysProProArgAsnTrpLysAspSerIleLeuThrLysAsnLeuIleLeuLysIleLeuValSerSerIleIleIleValCysGlyThrLeuPheValPheTrpArgGluLeuArgAsp
                    760                 770                 780                 790                 800
AATGTGATAACACCCCGAGACACAACAATGACTTTCACTTGCTTCGTGTTTTTTGATATGTTCAATGCACTGAGTTCCAGATCTCAGACTAAGTCTGTGTTTGAGATTGGACTCTGCAGTAATAAGATGTTCTGCTATGCGGTTCTTGGA  2550
AsnValIleThrProArgAspThrThrMetThrPheThrCysPheValPhePheAspMetPheAsnAlaLeuSerSerArgSerGlnThrLysSerValPheGluIleGlyLeuCysSerAsnLysMetPheCysTyrAlaValLeuGly
                    810                 820                 830                 840                 850
TCCATCATGGGGCAGTTGCTTGTTATCTACTTCCCGCCTCTGCAGAAGGTTTTTCAGACCGAGAGCCTCAGTATTCTGGATCTGTTGTTTCTTTTGGGTCTCACTTCATCAGTGTGCATTGTGTCTGAAATTATAAAGAAGGTTGAAAGG  2700
SerIleMetGlyGlnLeuLeuValIleTyrPheProProLeuGlnLysValPheGlnThrGluSerLeuSerIleLeuAspLeuLeuPheLeuLeuGlyLeuThrSerSerValCysIleValSerGluIleIleLeuLysLysValGluArg
                    860                 870                 880                 890                 900
AGCAGGGAAAAGACTCAGAAGAACACTACTTCAACACCATCGTCTTTTCTTGAAGTATGATGCATATTGCATTTTTTTTTTTCATTTGCAAACTAGGAATTGCAGTCTGGGAATCATTTAGAAGGGCAAGTTCAAGAGGATAATGAAGATT  2850
SerArgGluLysThrGlnLysAsnThrThrSerThrProSerSerPheLeuGluVal
                    910

TAAGGACTTTTAAACTTTCCATTGACTAAAAATGACCATTAATGTTAAAGACTTGATTTGAACCTTGTTGGTAGTCCCAAATGAAATTATGCAACTTTGAATAACACGTTCCTTGATTTGAATTGGCTTTTGCAATCGATTGGAACTTCA  3000
ACATATGGTCAAGTACTGAATTAAAAAAGTACAAAAGGCAAAACGTGAACACCTTCTGCACTTAGAGTTCTAATGTACAAATACACTATCTGTCTTAGATGGATATAAATATTTTTTTTATTTTTAAAATATTGTACTATTTATGGTTGTG  3150
GGGCTTTCTTACTAATACACTCAATAAATGCACTCAGTCATTTCAAAGGCATTCTATTTGGTTTAGAAATTGCCAGGAGTGCCATATTTCAGCTACCATGTTTCCTTTTTCCTGCAATGTACGCAAATGATACCTTGTGCTACCATTTTT  3300
ATATTTCAAGTTTTTTATTTTTTATTTTTGCACAGGATGTGACCACTGTCTAATCACTGTTCTTTCTTTTTGTGACTGAAAAGCATATACTGCAATTGAAGTAAATGTTTGCTCTTCTTAGTAAGTGTACATGGTTGTTTTGGTTTTTTG  3450
TAGTGGTTTTCCACAGAGGTGTGAGTCTTTGACCTAGCAGGATTAGTTCAGGCTGTTGTTGCATTACAGTTAGAGTAATTGTACTTAACACAGTCAAAGAGTCAAAAACAACAAAGGCCGGATGGAATTTCAAACAGACTATGTTAACAC  3600
GTCCTTGTTTAGGTTAATACAGTTCTACTTTGTCAGTGTCATCTTGTCATTTCTTCTTTATAAATCAAACACTTCAGCTAAATAATTGACCTTTATTACTGTTTCATATTTGTATTTTTAAGTCTATTTCTTAGAGAGTATAGACTACCA  3750
TACTTAGAAAATCAAAAGTCCCAAACTCAGAGAGATAGGACATGAACCAGGGTGTCCATCAGAAGATCCTGTGCAGGCCTTGCTCCTCCGAATCCTCTAGGCAGCCGCTTTGGCCTAGATTGTAGCACAACCCTGAGTCTGTCCCTTCTG  3900
CCGTTTGTTGGGATCCGTCTTCTTCTTTTGGTATGCTTGGGAAATAGATGGATTTGCTTTGTGTAAACCATCAGTTTTTATAAGAGCTTGACAAAATAAAGGTAAGTGTTGAATCTTGTATTTCATAAACTGTCAGAATTAACTGTTCTG  4050
AGCTTGTTCAATATTACTGGTGAGTGTTAACGCTGCTGCGCTGTCGTTGATAGGAGAACTCTGTTGTCTGAGTCTGTACTACTGAAGCATGAACGTAAGGAGCCAGATGTCTGACTTTGTCTTAATCTCTCAGGCTCAACTGTCAGTGTA  4200
CATTGTTCTGTCTCATTAGGTGGGACGTGAGGTCACTGTCAACCAACTCATAGAGGTTTGATGTCTCATAGCAAAATTTTATAATGGTTTTTTACATTCTGTTCCAAACTTGAAAACTACATATTAAGTGCATATTCAGTCTTGTTTGTT  4350
TTACAGTAGAGGTTGAGAATATGACTCATGAAATGAATGTGCCTATCTTTTCACTTGGTATTGAACTATAAAAATAAAGTGAGTTTTTCATAAGTTCAAAAAAAAAAAAAAAAAAAA  4467
```

FIGURE 2: Composite nucleotide sequence of rat stomach cDNA RS10-31 and rat testis cDNA RT7-1 and deduced amino acid sequence of the protein. The nucleotides are numbered to the right of the sequence with nt +1 corresponding to the probable translation initiation site. Amino acids are numbered below the sequence. Two polyadenylation signals that seem to be functional (see text) are underlined. The phosphorylation site (Asp-350) is underlined and labeled. Stomach cDNA RS10-31 begins at nt −178 and terminates at nt 3655 while testis cDNA RT7-1 begins at nt 2138 and terminates with the poly(A) tract.

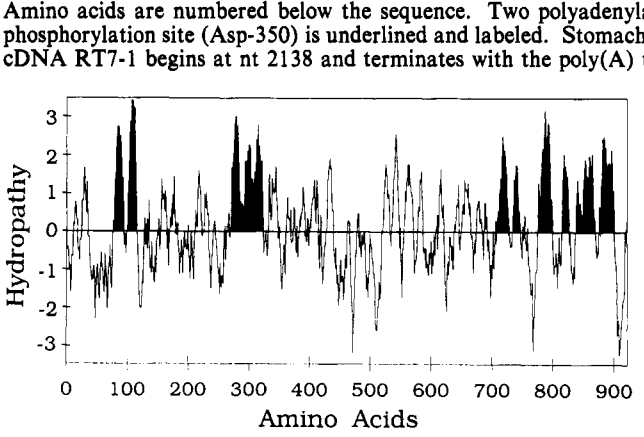

FIGURE 3: Hydropathy profile of the protein encoded by stomach cDNA RS10-31. Hydropathy values were determined by the procedure of Kyte and Doolittle (1982) using a window of 9 amino acids. Possible membrane-spanning regions are darkened.

3999 is utilized.

In testis, the 3′ untranslated sequence probe detected low levels of the 3.9- and 5-kb mRNAs, but the major transcript detected with the coding sequence probe was a 3.7-kb mRNA that did not appear to be expressed in other tissues. The 3′ untranslated sequence probe from clone RS10-31 did not hybridize with the 3.7-kb mRNA. This raised the possibility that the 3.7-kb mRNA is an alternatively spliced transcript encoding a testis-specific variant of the protein. S1 nuclease protection analysis of rat testis and stomach poly(A)$^+$ RNAs (data not shown) indicated that the sequence of the testis-specific mRNA diverged from that of the stomach mRNA just prior to the stop codon. This suggested that the testis-specific mRNA encoded a protein with an alternative C-terminus.

Isolation and Characterization of Rat Testis cDNAs Encoding the Putative Housekeeping and Testis-Specific Forms of the Protein. To isolate cDNAs for the alternative transcript in testis, a rat testis library was screening with a cDNA probe from the coding sequence of clone RS10-31. Ten phage plaques were identified and pulled from the master plates. The pBluescript phagemids containing the cDNA

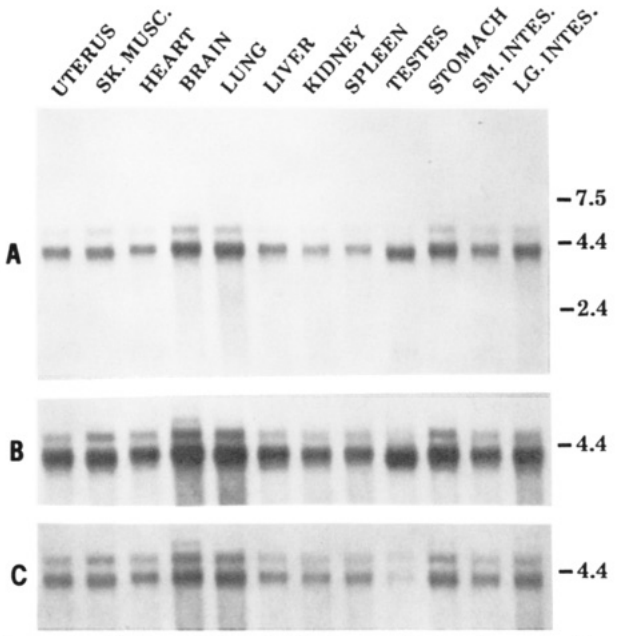

FIGURE 4: Northern blot hybridization analysis using probes from clone RS10-31. Poly(A)$^+$ RNA from the indicated rat tissues was analyzed using probes from the 3′ untranslated region (3′ probe) and coding sequence (5′ probe) of stomach cDNA RS10-31 as described under Experimental Procedures. The probe and autoradiographic exposure times were (A) 5′ probe, 26 h; (B) 5′ probe, 66 h; (C) 3′ probe, 6.5 day. The positions and sizes (in kilobases) of the RNA markers are shown on the right.

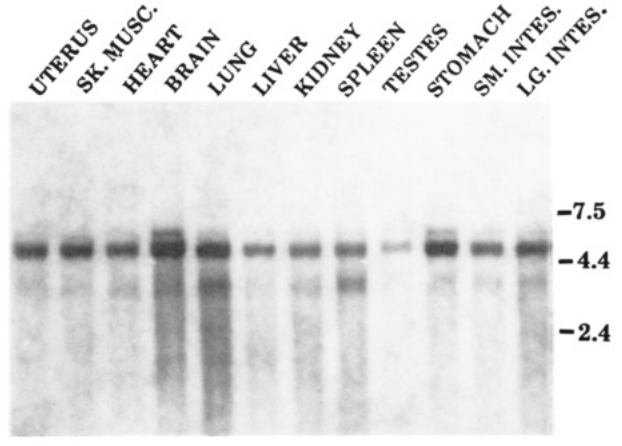

FIGURE 5: Northern blot hybridization analysis using a probe from the 3′ end of testis cDNA RT7-1. Poly(A)$^+$ RNA from the indicated rat tissues was analyzed using a probe from the extreme 3′ end of clone RT7-1 as described under Experimental Procedures. The autoradiographic exposure time was 8 days. The positions and sizes (in kilobases) of the RNA markers are shown on the right.

inserts were excised from the λ Uni-ZAP vector, colony purified, and analyzed by restriction endonuclease mapping, Southern blot hybridization, and DNA sequence analysis. One cDNA lacked the C-terminal coding sequence and, therefore, could not be classified. Three partial cDNAs, including clone RT7-1, encoded an enzyme with the same C-terminus as that encoded by stomach cDNA RS10-31. As mentioned above, Northern blot hybridization analysis using a cDNA probe from the 3′ end of RT7-1 (Figure 5) demonstrated that it was derived from the 5-kb mRNA seen in all tissues.

DNA sequence analysis of the remaining six clones demonstrated that they were partial cDNAs derived from the alternatively spliced testis mRNA. The restriction maps for two of these clones, RT13-1 and RT15-1, which were used to determine the sequence of the testis-specific transcript, are shown in the lower part of Figure 1. The sequence corresponding to the alternative 3′ coding and untranslated sequence was also determined for the four additional clones. The nucleotide and deduced amino acid sequences immediately preceding and following the point of divergence are shown in Figure 6. The point of divergence between the two cDNA classes occurred between codons 918 and 919 (arrow in Figure 6). The alternatively spliced testis mRNA encodes a protein in which the last residue of the putative housekeeping form of the enzyme, Val919, is replaced by the short amino acid sequence Phe-Tyr-Pro-Lys-Ile. The coding sequence was followed by a 3′ untranslated sequence that differed slightly in length among the six cDNAs examined. Two apparent polyadenylation sites, separated by about 140 nts, were observed. Several cDNAs had extensive poly(A) tracts following nts 3173 or 3176, and one cDNA had a poly(A) tract at nt 3315. An additional cDNA contained sequences that extended well beyond nt 3315, but Northern blot analysis with a probe derived from the extra sequence failed to identify an mRNA species (data not shown) suggesting that the extra sequence was a cloning artifact. Just upstream of each of the polyadenylation sites are sequences which are similar, but not identical, to the consensus polyadenylation signal (underlined in Figure 6).

```
2701                                                    ↓
AGCAGGGAAAAGACTCAGAAGAACACTACTTCAACACCATCGTCTTTTCTTGAATTTTAC
SerArgGluLysThrGlnLysAsnThrThrSerThrProSerSerPheLeuGluPheTyr
                                  910                   920
CCCAAGATTTAATCATCTTAGCTGTCCAGAACTCCTTTTGTTACTTGAACTACTAATTGA
ProLysIle
      923
AGATTGACGTGTCCAAAGCCTCAGGTTTGGCTCTGGCAGAGGAGTGGACAGCAGCTGATT

GAGATACAAGCCCATCTGGAGACAGGACTGCCACTGACAGAAGACATGGGCTGTGTCTAA

ATCCAATCTGGGACCCGGCCGCATGGCTGCCTTCACCTGTTGTAATTCTTCATACAGCAT

CTTGGCACCACCTCTCCGTGGATCTTCACTATCTACGTAAAACAACCCAAAGACAGCGTT

TCCAGACACTGCATCAGTTTGCCCTTCCCCACATTCCATCATCAAAGCCACAAAGACAAT
                                                      * *
CAGTGTGAAAACTGGTCACTTTTCTTTTCTGGTTTCATAAATGTCTTCCATACACTGAAG

GTCCCTTTTGACTTTATTCAACCCAAAGTTACTGATTTTTCCTTAAAATAAGATCTCTCC

CATAGTATGCTACCTGACCTTAAACAAGTACATTGTCTAGGATCCGAGTCTATGAAAATA

ATGACACTAAACACC-poly(A) tract
      3315
```

FIGURE 6: Nucleotide sequence from the 3′ end of the testis-specific cDNA. The nucleotide sequences of the transcript expressed in all tissues and the testis-specific transcript are identical up to nt 2754. The sequence shown begins at nt 2701 just before the point at which the two transcripts diverge (indicated by a vertical arrow). The protein encoded by the testis-specific mRNA has an alternative 5 amino acids in place of the last amino acid of the form expressed in all tissues (see Figure 2). Amino acids are numbered below the sequence. Asterisks are shown above the nucleotides that were followed by extensive poly(A) tracts in several cDNAs. A single cDNA, clone RT15-1, had a poly(A) tract following nt 3315. Sequences that may serve as polyadenylation signals are underlined.

To confirm that the cDNAs with the alternative 3′ end correspond to the 3.7-kb mRNA in testis, Northern blot hybridization was performed using a probe from the unique 3′ untranslated sequence. As shown in Figure 7, the only mRNA identified was the 3.7-kb testis mRNA, which was not detected in any other tissue. Thus, the 3.7-kb mRNA, which encodes a variant of the enzyme that has an alternative C-terminus, appears to be expressed only in testis.

The Novel P-Type ATPase Exhibits High Sequence Similarity to the Yeast Secretory Pathway Ca^{2+} Pump and Other Ca^{2+}-ATPases. Similarity comparisons demonstrate that the amino acid sequence of the P-type ATPase identified in this study most closely resembles those of a yeast secretory pathway Ca^{2+}-ATPase (Rudolph et al., 1989) and the SERCA

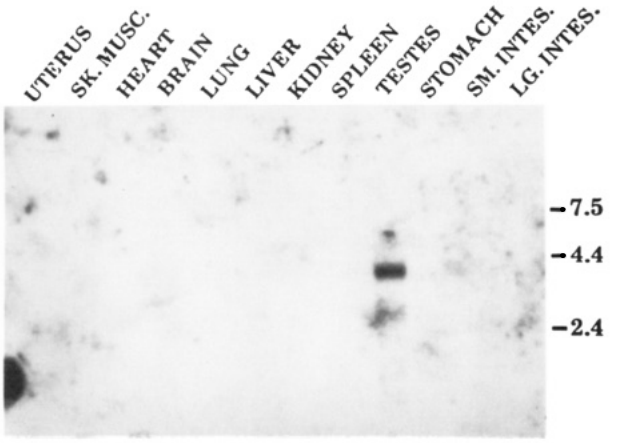

FIGURE 7: Northern blot hybridization analysis using probes corresponding to the testis-specific mRNA. Poly(A)$^+$ RNA from the indicated rat tissues was analyzed using a cDNA probe from the 3′ untranslated region of testis cDNA RT5-1 as described under Experimental Procedures. The autoradiographic exposure time was 8 days. The positions and sizes (in kilobases) of the RNA markers are shown on the right.

and PMCA classes of mammalian Ca^{2+} pumps. Amino acid similarity comparisons between the protein encoded by clone RS10-31, the yeast secretory pathway Ca^{2+} pump (termed PMR1), SERCA2a, and PMCA1a are shown in Figure 8. In pairwise comparisons, it exhibits 23% amino acid identity to PMCA1a, 33% identity to SERCA2a, and 50% identity to the secretory pathway Ca^{2+} pump. It should be noted that the amino acid identity between SERCA2a and PMCA1a, which are members of two different classes of mammalian Ca^{2+} pumps, is only 27%. Thus, the degree of similarity observed between the different enzymes is consistent with the possibility that the novel ATPase represents a third distinct class of mammalian Ca^{2+} pump.

The highest degree of amino acid similarity among the four pumps occurs in the major cytoplasmic regions between the second and third transmembrane domains and between the fourth and fifth transmembrane domains. These conserved regions are involved in basic catalytic activities of the P-type ATPases such as ATP binding, phosphorylation, and conformational transitions of the enzyme. Predicted transmembrane domains 4 and 6 also exhibit a relatively high degree of similarity among the four pumps. These putative transmembrane domains contain 4 of the 6 residues (Glu-309, Glu-770, Asn-795, Thr-798, Asp-799, and Glu-907 in SERCA2a) that seem to form the high-affinity Ca^{2+} binding sites in the SR Ca^{2+}-ATPases (Clarke et al., 1989). The two additional residues known to be involved in Ca^{2+} binding in the SR Ca^{2+} pump are in putative transmembrane domains 5 and 8. Three of the six residues, Glu-308, Asn-738, and Asp-742 of the protein encoded by RS10-31, are conserved in all four pumps. Glu-770 and Thr-798 of SERCA2a correspond to Ala and Met residues, respectively, in the other pumps. Glu-907 of SERCA2a corresponds to Asp residues in the RS10-31 protein and PMR1 and to Gln in PMCA1a.

The RS10-31 protein and PMR1 exhibit a striking degree of amino acid similarity throughout their lengths. Extended regions of perfect identity between the rat and yeast proteins occur in the catalytic domains and in predicted transmembrane domains 2, 4, 5, 6, and 8. The amino acid identity within these predicted transmembrane domains is 84%, much greater than the overall identity. A significant degree of identity also occurs in the remaining transmembrane domains, in the N-terminal and C-terminal regions, and in the short sequences connecting some of the transmembrane domains. In aligning the RS10-31 protein with PRM1 it was not necessary to introduce extended gaps, whereas extensive gapping was necessary in order to maintain the alignment with SERCA2a and PMCA1a. The overall similarity in amino acid sequence and general organization strongly suggests that the rat and yeast enzymes are functional homologues.

DISCUSSION

In an earlier study (Gunteski-Hamblin et al., 1988), we identified a partial cDNA encoding a P-type ATPase that was more closely related to the SERCA and PMCA Ca^{2+} pumps than to other mammalian P-type ATPases. In order to determine the complete amino acid sequence of the protein, and to obtain information regarding its tissue distribution, we carried out additional cDNA cloning studies and Northern blot hybridization analyses. A stomach cDNA containing the complete coding sequence was isolated and characterized, and Northern blot analyses demonstrated that the corresponding mRNAs are expressed in all tissues, suggesting that the enzyme plays a housekeeping role. Interestingly, a testis-specific mRNA was also identified that seemed to represent an alternatively spliced transcript of the same gene. By isolating and characterizing the corresponding cDNA, we determined that the testis-specific mRNA encodes a variant of the protein that contains an alternative C-terminus.

Similarity comparisons reveal that the enzyme encoded by clone RS10-31 is more closely related to several known Ca^{2+} pumps than to other P-type ATPases. It exhibits 23% amino acid identity with the plasma membrane Ca^{2+}-ATPase, 33% identity with the SR Ca^{2+}-ATPase, and 50% identity with PMR1, the yeast secretory pathway Ca^{2+}-ATPase (Rudolph et al., 1989). Similarity between the RS10-31 protein and PMR1 occurs throughout the two polypeptide chains. Extensive amino acid identity occurs not only in the catalytic domains, which are conserved among all P-type ATPases, but also in the hydrophobic sequences corresponding to the 10 putative transmembrane domains identified in other pumps [discussed in MacLennan et al. (1985), Shull and Greeb (1988), and Clarke et al. (1990)]. These domains are poorly conserved between pumps that differ in their ion specificities. If all ten potential transmembrane domains are considered, the amino acid identity between the two proteins is 63%, considerably greater than the overall identity of 50%. The identity within the predicted transmembrane domains corresponding to those that contain the Ca^{2+} binding residues in the SR Ca^{2+}-ATPase is greater than 80%. These transmembrane domains are also relatively conserved between the RS10-31 protein and SERCA2a (44% identity in the transmembrane domains vs 33% overall) or PMCA1a (38% identity in the transmembrane domains vs 23% overall). On the basis of these comparisons, it seems likely that the protein encoded by clone RS10-31 is a Ca^{2+}-transporting ATPase and that it is the mammalian homologue of the yeast secretory pathway Ca^{2+} pump. If this proves to be the case, then it represents a third distinct class of mammalian Ca^{2+} pump and would most likely function in maintaining Ca^{2+} concentrations within one or more compartments of the secretory pathway.

PMR1 was classified as a Ca^{2+} pump on the basis of its similarity to known Ca^{2+}-ATPases and the observed effects of Ca^{2+} on the phenotype of pmr1 mutants (Rudolph et al., 1989). Null mutants of the PMR1 gene grow poorly on solid medium containing 1 μM Ca^{2+} but grow well on medium containing 20 mM Ca^{2+}. They also exhibit hypersensitivity to EGTA and the anti-calmodulin drug trifluoperizine, and these sensitivities are overcome by addition of Ca^{2+}. Compared

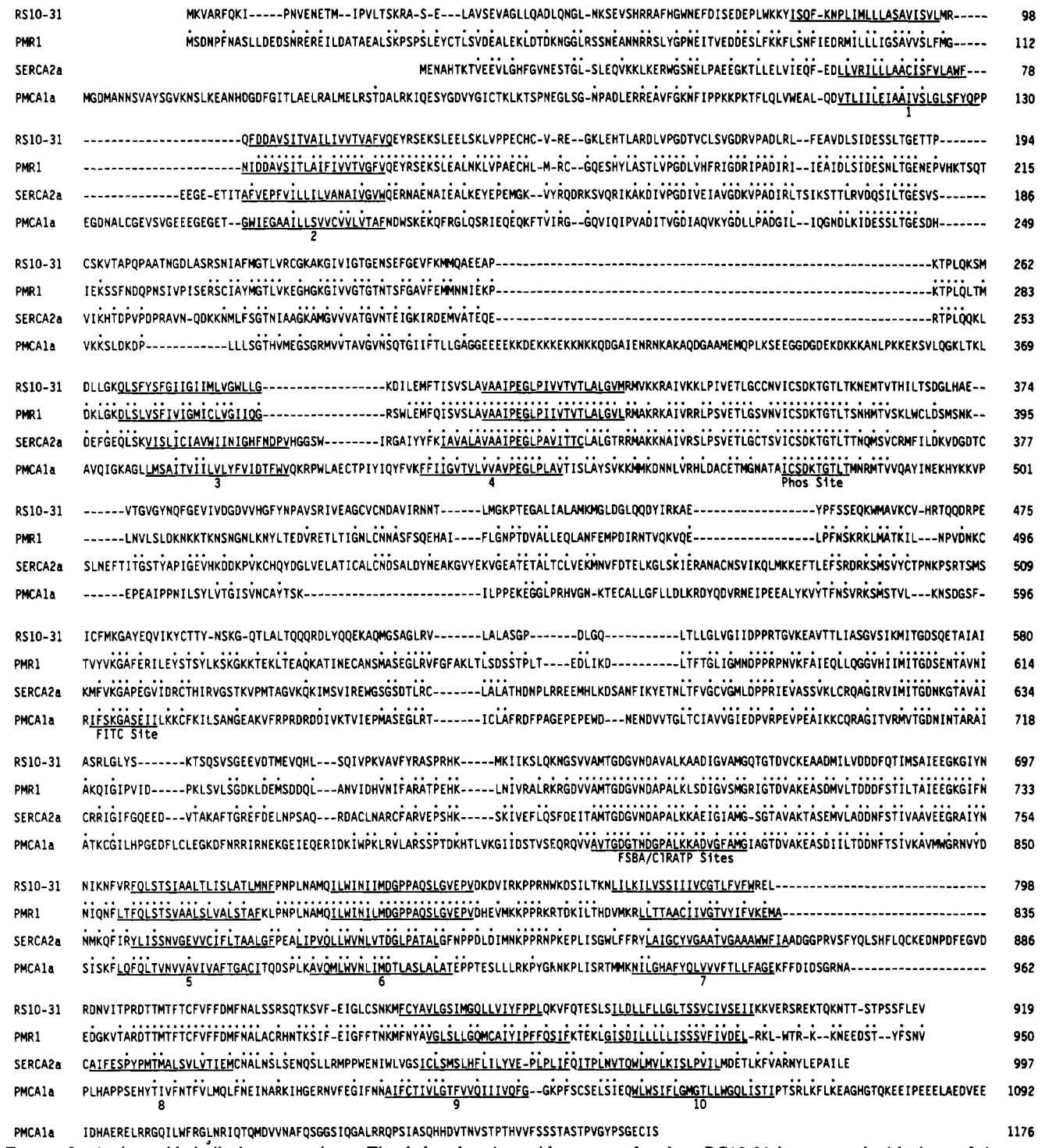

FIGURE 8: Amino acid similarity comparison. The deduced amino acid sequence for clone RS10-31 is compared with those of the yeast secretory pathway Ca^{2+} pump, termed PMR1 (Rudolph et al., 1989), the cardiac SR Ca^{2+}-ATPase (SERCA2a) (MacLennan et al., 1985), and the plasma membrane Ca^{2+}-ATPase isoform 1a (PMCA1a) (Shull & Greeb, 1988). Gaps (indicated by dashes) were introduced to maintain the alignment. Dots above amino acids of PMR1, SERCA2a, or PMCA1 indicate identity to the corresponding residue in the protein encoded by clone RS10-31. Hydrophobic regions which may represent transmembrane domains [identified by the procedure of Eisenberg et al. (1984)] are underlined and numbered. The phosphorylation domain (Phos Site), FITC binding site (Farley et al., 1984; Mitchinson et al., 1982; Filoteo et al., 1987), and the region which binds FSBA (Ohta et al., 1986) and ClRATP (Ovchinnikov et al., 1987) in the Na,K-ATPase are underlined. Amino acid numbers are shown on the right.

with wild-type cells, pmr1 mutants have been shown to secrete much higher levels of heterologous proteins such as bovine prochymosin, bovine growth hormone, and a variant of human urinary plasminogen activator (Smith et al., 1985). Outer chain glycosylation is also defective in pmr1 mutants; invertase from pmr1 strains contains core polysaccharide but lacks the outer mannose chains (Rudolph et al., 1989), which are added to the core polysaccharide in the Golgi. In their original study describing the cloning of PMR1, Rudolph et al. (1989) suggested that the aberrant secretion and glycosylation seen in pmr1 mutants could result from a failure to sequester Ca^{2+}, with subsequent alterations in cytosolic Ca^{2+} concentrations or, alternatively, that PMR1 might function directly within a compartment of the secretory pathway. Using immunocytochemical and subcellular fractionation techniques, Antebi and Fink have recently shown that PMR1 is located in

a Golgi-like organelle, confirming that PMR1 functions in a compartment of the secretory pathway.[2]

A number of investigators have identified a Ca^{2+} pump in Golgi membranes of rat (West, 1981; Virk et al., 1985) and mouse (Watters, 1984; Neville et al., 1981) mammary glands. The enzyme is sensitive to vanadate (Virk et al., 1985), indicating that it is a P-type ATPase. Activity of the pump appears to be at least partly dependent on the existence of a pH gradient across the Golgi membrane (acid interior) (Virk et al., 1985), suggesting that it might transport Ca^{2+} in exchange for H^+ as in the case of the plasma membrane Ca^{2+}-ATPase (Niggli et al., 1982; Smallwood et al., 1983). The protein encoded by clone RS10-31, which seems to be expressed in all tissues, is a likely candidate for this pump. If the RS10-31 protein and the testis-specific variant are Golgi Ca^{2+} pumps, it is possible that they are not restricted to this compartment. Fasolato et al. (1991) have identified a large acidic intracellular Ca^{2+} pool in PC12 cells that is insensitive to thapsigargin, an inhibitor of the SERCA class of Ca^{2+} pumps (Lytton et al., 1991). In PC12 cells, this pool consists primarily of secretory granules, but other acidic organelles, including lysosomes, endosomes, and Golgi vesicles might contribute to the pool. Immunocytochemical studies will be needed to determine whether the pumps cloned in our study are located in the Golgi and, if so, whether they might also be present in other organelles of the secretory pathway.

The testis-specific isoform of the enzyme has a C-terminus in which the last residue of the RS10-31 protein is replaced by an alternative 5-amino acid sequence. Alternative splicing of exons encoding C-terminal sequences is a common occurrence among Ca^{2+}-ATPase genes (Strehler et al., 1989; Brandl et al., 1987; Lytton & MacLennan, 1988; Gunteski-Hamblin et al., 1988). It has been suggested that the alternative C-termini of the SERCA pumps might function as sorting signals (Burk et al., 1989; Lytton & MacLennan, 1988) but, so far, there have been no rigorous experimental tests of this hypothesis. In the case of the PMCA pumps, alternative splicing is known to alter regulatory domains near the C-terminus (Strehler et al., 1989). It will be of interest to determine which cell type(s) in testis express the alternative 923 amino acid isoform. We are currently performing in situ hybridization analysis to address this question. A likely possibility is the Sertoli cell, which is involved in forming the blood testis barrier and serves as the major secretory cell of the seminiferous epithelium (Bardin et al., 1988; Dym, 1977).

Using the procedures described by Maruyama and MacLennan (1988), we have made a number of attempts to determine directly whether the RS10-31 protein is a Ca^{2+} pump, but so far, these experiments have given negative results (data not shown). In our first set of experiments, we transfected COS-1 cells with constructs containing the wild-type coding sequence but were unable to detect increased Ca^{2+} uptake in microsomes. Additional experiments were performed using constructs in which the C-terminal coding sequence for the RS10-31 protein had been replaced by that of SERCA3, which resembles an ER retention signal (Burk et al., 1989). We hoped that this approach would allow retention of the protein in the ER and that this would allow an increased expression of Ca^{2+} uptake activity; however, no increase in Ca^{2+} uptake was detected. The reason for the lack of Ca^{2+} pump activity in these experiments is unclear. Because the appropriate antibodies are not yet available, we have been unable to determine whether the protein is being expressed in our transfection studies. It is conceivable that a second subunit is required for activity, although all of the other Ca^{2+} pumps that have been identified function as a single polypeptide. A more likely possibility is that the conditions used in this study, which are appropriate for measurement of the activity of the SERCA pumps, are not appropriate for detecting activity of the secretory pathway pump.

In conclusion, we have identified and characterized rat cDNAs encoding possible homologues of the yeast secretory pathway Ca^{2+} pump and have determined their mRNA tissue distribution. Questions concerning the membrane location of these pumps, their biological roles, and whether they do, in fact, function as Ca^{2+}-transporting ATPases remain to be determined. To address the latter question, which is central to our understanding of the biological role of these enzymes, it may be necessary to obtain information about the Ca^{2+} uptake activity of the compartment in which these proteins normally reside, as this should enable the development of a more appropriate assay system. The recent study of Fasolato et al. (1991) may have some relevance in this regard. The acidic Ca^{2+} pool that they identified is quite stable; it does not appear to release Ca^{2+} at a significant rate and the rate of Ca^{2+} uptake into the pool is very low. On the basis of these observations, they suggested that it may represent a dead-end Ca^{2+} pool that does not contribute to short-term fluctuations in cytosolic Ca^{2+} levels that occur during signaling events. If the RS10-31 protein is the pump which serves this pool, then measurement of its activity may require the use of procedures which deplete intravesicular Ca^{2+} prior to conducting Ca^{2+} uptake assays.

[2] Gerald R. Fink, personal communication.

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