J. Euk. Microbiol., 44(3), 1997 pp. 250–257

Cloning and Molecular Analysis of the Plasma Membrane Ca^{2+}-ATPase Gene in *Paramecium tetraurelia*

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ABSTRACT. We have determined the DNA sequence of the gene encoding the protein of the plasma membrane Ca^{2+}-ATPase in *Paramecium tetraurelia*. The predicted amino acid sequence of the plasma membrane Ca^{2+}-ATPase shows homology to conserved regions of known plasma membrane Ca^{2+}-ATPases and contains the known binding sites for ATP (FITC), acylphosphate formation, and calmodulin, as well as the "hinge" region: all characteristics common to plasma membrane Ca^{2+}-ATPases. The deduced molecular weight for this sequence is 131 kDa. The elucidation of this gene will assist in the studies of the mechanisms by which this excitable cell removes calcium entering through voltage gated calcium channels and the pump functions in chemosensory signal transduction.

Supplementary key words. Ca^{2+}-ATPase, calcium, ciliate, homeostasis.

THE plasma membrane Ca^{2+} pump is an enzyme that plays an important role in controlling the concentration of free intracellular Ca^{2+} in all eukaryotic cells studied thus far. It is the largest of all P-type ATPases [23] with a molecular weight between 128–150 kDa [34]. Plasma membrane Ca^{2+} pumps constitute a multigene family which currently consists of four known genes [10, 11, 36] and additional isoforms which are the result of alternative RNA splicing [10, 11, 36]. Members of the gene family have been cloned from a variety of tissues of higher organisms including: human erythrocytes, teratoma cells, intestine, smooth muscles of rabbit and pig, and rat brain [5, 10, 11, 14, 33, 36, 38]. Even with such diverse origins, all known isoforms for the plasma membrane Ca^{2+} pump contain sequences for sites of ATP (fluorescein isothiocyanate site [FITC]), calmodulin, Ca^{2+} binding, and acylphosphate formation [5, 10, 14, 33, 35, 36, 38].

In *Paramecium*, when the membrane is depolarized in response to mechanical or ionic stimuli, Ca^{2+} enters the cell through voltage-gated Ca^{2+} channels located on the cilia [21, 27]. In contrast to these well studied Ca^{2+} channels, little is known about the mechanism responsible for reducing intracellular Ca^{2+}. Browning and Nelson [2] demonstrated Ca^{2+} is continuously expelled from *Paramecium* by a temperature-dependent mechanism they suggested was an ATP driven pump. Wright and Van Houten [40] have reported characteristics of a Ca^{2+}-ATPase and a Ca^{2+}-dependent phosphoprotein found in the pellicle (plasma membrane plus tightly bound underlying alveolar sacs) of *Paramecium* and suggested this to be a plasma membrane Ca^{2+} pump. Additionally, Wright et al. [41] reported a protein of appropriate molecular weight was present in the pellicle and bound calmodulin, a characteristic found in all plasma membrane Ca^{2+}-ATPases [5, 10, 14, 33, 35, 36, 38].

We report here the DNA sequence of a Ca^{2+} pump gene in *Paramecium tetraurelia* with a significant homology between this sequence and known plasma membrane Ca^{2+} pumps. Additionally, this sequence contains the known binding sites for ATP (FITC site), acylphosphate formation, a short calmodulin binding domain, and a deduced molecular weight of 131 kDa.

MATERIALS AND METHODS

Cell culture. *Paramecium tetraurelia,* 51-S (sensitive to killer), were grown in culture medium as described by Sasner and Van Houten [31].

***Paramecium* genomic DNA isolation.** Genomic DNA was prepared by the protocol of Forney and co-workers [9].

Initial primer synthesis. All primers needed for polymerase chain reaction (PCR) and sequencing (except the T_3 and T_7 primers from Stratagene, San Diego, CA) were made in an Applied Biosystems DNA (Foster City, CA) synthesizer model 391.

Cloning of polymerase chain reaction products. The first sets of primers were designed to amplify an internal segment of P type pump genes. The "hinge" conserved amino acid region for all ion transporting ATPases, GTNDGPAL found at amino acid #797–804 for human red blood cell plasma membrane Ca^{2+} pumps [38], was used to design the 5′ forward primer 5′AC(A/T)GATGGATCCAATGATGGACCAGC(T/A)TTAAA3′. The reverse 3′ primer 5′ATCCTCGAGCAAATTAACCCA(T/C)AACATTTAAC3′ was based on VQMLWVNL, found at amino acids #885–895 for human red blood cell plasma membrane calcium pumps [38]. This is a conserved amino acid region for plasma membrane and sarcoplasmic reticulum Ca^{2+}-ATPase that is thought to play a role in moving the Ca^{2+} ion across the membrane [38]. The sequence for each oligonucleotide primer was based on the codon usage frequency for genes sequenced in *Paramecium* [19], with the addition of sequences for 51A and 51C surface antigens [24, 25], α-tubulin (L. Spitzer, unpubl. data), and calmodulin (R. Hinrichsen, pers. commun. and [15]). Restriction enzyme cut sites were also incorporated into each primer. The 5′ forward primer had a *Bam H* I cut site designed into the primer. The 3′ reverse primer was designed with a *Xho* I cut site present.

Amplification of the genomic sequence was carried out according to the basic PCR protocol [4] (GTG-1 Genetic Thermal Cycler from Precision Scientific, Bloomington, IN).

Once the PCR product was isolated and purified, it was resuspended in 10 μl dH_2O, cut with the restriction enzymes (BRL, Baltimore, MD, now Gibco/BRL, Gaithersburg, MD) using the BRL buffers, ligated into Bluescript (Stratagene, San Diego, CA) and used to transform XL-blue competent cells using the protocols in [32]. (Each restriction enzyme used was dictated by the cut site built into the primer. See Table 1 for primer sequences.)

Plasmid preparation and sequencing. After the transformation, the plasmid DNA with insert was prepared for sequencing with the Magic Minipreps DNA Purification System (now called Wizard Minipreps [Promega, Madison, WI]). The double-stranded plasmids were converted to a single stranded form prior to sequencing by alkali denaturation.

The protocols recommended by the manufacturer of Sequenase Version 2.0 (U. S. Biochemicals, Cleveland, OH) were followed. Sequencing was done by the dideoxy chain termination method employing [α-^{35}S] ATP (Amersham, Arlington Heights, IL). Sequencing of both strands of two PCR products was carried out.

DNA labeling and Southern transfers. Genomic DNA (5 μg/reaction) was cut with the following restriction enzymes: *Bcl*

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I, *Eco R* I, *Kpn* I, *Hind* III, *Mlu* I, *Nsi* I, *Sst* I, *Xba* I, *Cla* I, *Pst* I, *Pvu* II, and *Hae* III (BRL).

DNA was labeled with digoxigenin-11-dUTP following the random primer method from the Genius I system (Boehringer Mannheim, Indianapolis, IN). Southern blot gel, transfer, prehybridization and hybridization were done according to Amersham's *Protocols for Nucleic Acid Blotting and Hybridization* and Boehringer Mannheim Biochemicals protocol for the Genius kit.

Inverse PCR. Inverse PCR procedures were used to move upstream and downstream from the original sequenced PCR product. The genomic DNA was cleaved with *Cla* I, *Eco R* I, *Hae* III, or *Xba* I (BRL). The genomic DNA (7 μg/reaction) was digested with ten units of each restriction enzyme overnight at 37° C. After the overnight digestion, DNA was extracted with phenol, phenol/chloroform, and chloroform. The final aqueous phase was precipitated with 1/10 volume of cold 3 M Na acetate (pH 5.2) and 100% EtOH overnight at −70° C. The DNA pellets were washed twice with EtOH, dried under vacuum. Each pellet was resuspended in 1.25 ml sterile dH_2O (yielding a final concentration of less than 2 μg/ml) to which 250 μl T_4 DNA ligase buffer (6X, BRL) and 20 μl (1 μl/unit) T_4 DNA ligase (BRL) were added [20]. The reaction was incubated overnight at 15° C and later stored at 4° C.

The circularized DNA molecules were precipitated with ethanol and 3 M Na acetate (pH 5.2). After washing the pellet with EtOH to remove salt, the pellets were resuspended in 50 μl dH_2O, and the concentration confirmed using a fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Two inverse PCR primers (Table 1) were designed from the original sequenced PCR product (285 bases), these two primers could be used on genomic DNA cut with *Hae* III or *Xba* I. After the 2.5 kb and 1.2 kb inverse PCR products from genomic DNA cut with *Hae* III, and *Xba* I were sequenced, 2 additional sets of inverse PCR primers (Table 1) were designed. From the Southern blot results, we predicted inverse PCR products of 1.5 and 2.5 kb when the genomic DNA was cut with *EcoR* I or *Cla* I respectively and amplified with the inverse primers in Table 1.

Ligation of inverse PCR products. All the inverse PCR primers used contained restriction enzyme cut sites (Table 1). Once cut, the PCR products were isolated and purified, and ligated with pBluescript plasmid (Stratagene, San Diego, CA).

Full length PCR amplification and sequencing strategy. The primers used for the PCR amplification of the full-length plasma membrane Ca^{2+} pump were designed and based upon the primary plasma membrane Ca^{2+} pump sequence from *Paramecium tetraurelia.* Each primer contained an artificial restriction enzyme cut site toward its 5′ end to allow for insertion of the full length PCR product into the pBluescript KS^+ plasmid. The 5′ forward primer was designed from the initial sequence located 70 bp 5′ to the start codon. This primer included a *Kpn* I cut site:

5′ CCCGGTACCGTGTTCTCAGATTCATT 3′

The 3′ reverse primer was designed from the sequence 155 bp downstream from the stop codon. This primer contained a *Hind* III cut site:

5′ GGTAAGCTTAACATTACACC 3′

PCR amplification was performed using the following conditions: 1.5 mM $MgCl_2$, 50 μM dNTP, 0.5 μM primers (listed above), 500 ng genomic DNA, and 2.5 U *Taq* polymerase (BRL). Thirty cycles of denaturation (94° C, 1 min) primer annealing (52° C, 2 min) and extension (72° C, 3 min) were used for the amplification.

The PCR product was inserted into the pBluescript KS^+ plasmid using the incorporated cut sites designed within each primer. The same procedures listed above under ligation, transformation, plasmid prep, and sequencing were utilized. Sequence analysis was done using both the MacVector (Kodak, Rochester, NY) and Genetic Computer Group, Inc. (GCG, Madison, WI) computer programs. GCG Accession Number: U05880.

RNA isolation. RNA isolation was based on a modified hot phenol extraction method [22]. mRNA was isolated by using an mRNA selection kit (5 prime → 3 prime, Boulder, CO).

Formaldehyde denaturing electrophoresis gel and Northern blotting. Both the total RNA and mRNA were pelleted in ethanol and centrifuged for 30 min at 4° C, rinsed twice with 70% ice cold ethanol and dried. All the samples were resuspended in 22 μl loading buffer (4.5 μl H_2O, 2 μl 5× MOPS buffer (0.1 M MOPS, 5 mM EDTA, pH 8.0, 40 mM Na acetate) 3.5 μl formaldehyde, 10 μl formamide, and 2 μl 0.5% bromophenol blue and xylene cyanol). The samples were heated at 65° C for 15 min prior to loading, then cooled on ice for 1–2 min prior to loading. While the samples were being incubated at 65° C, a 1% agarose gel (2.3 M formaldehyde, 20 mM MOPS (pH 7.4), 1.6 mM EDTA (pH 8.0)) was pre-warmed for 15 min at 65 V prior to loading. RNA markers (BRL) and total RNA were loaded in addition to the mRNA. Gels were run in a 1× buffer (20 mM MOPS, 1 mM EDTA) for ≈ 3 h at 65 V.

When electrophoresis was completed, the gel was cut into identical halves for staining and for transfer overnight to nitrocellulose (Schleicher & Schuell, Midwest Scientific, St. Louis, MO) using the Turbo blotter (Schleicher & Schuell) with 20X SSC. The membrane was baked under vacuum for 2 h at 80° C.

Labeling of probe, hybridization, and signal detection. The final 900 bases of the plasma membrane Ca^{2+}-ATPase DNA sequence was amplified by PCR, run on a 1% agarose gel, excised from the gel and purified using *Gene Clean* (Bio 101, Midwest Scientific, St. Louis, MO). This portion of the gene would be used as the probe for the plasma membrane Ca^{2+}-ATPase mRNA. The DNA (100 ng) was denatured by boiling, then labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA) using the Random Primers Labeling kit (BRL). Prehybridization, hybridization, and autoradiography were carried out by the protocols of Sambrook and others [29].

RESULTS

Initial amplification of a conserved region for plasma membrane Ca^{2+}-ATPase. The initial primers used to amplify genomic DNA were designed from two highly conserved regions. The first conserved region used for the 5′ primer was based on the "hinge" region, which is conserved for all P-type ATPases, and corresponds to amino acids #797–804 in human red blood cell plasma membrane Ca^{2+} pumps [38]. The 3′ primer was designed from the conserved amino acid region that is thought to play a role in moving the Ca^{2+} ions across the membrane [38], and corresponds to amino acids #885–895 in the human red blood cell plasma membrane Ca^{2+} pumps [38]. The *Paramecium* codon usage, based on previously cloned genes, was taken into account in the design of degenerate primers. The initial PCR results showed a product within the expected size of 285–290 bp.

The degenerate primers used for the initial PCR amplifications contained restriction enzyme sites for *Bam H* I or *Xho* I. The 285 bp PCR products were digested and directly ligated into the KS^+ pBluescript plasmid. Plasmid DNA from six transformed clones was sequenced and analyzed. A clone with the deduced amino acid sequence that was highly homologous to plasma membrane Ca^{2+}-ATPases was used further. (Other

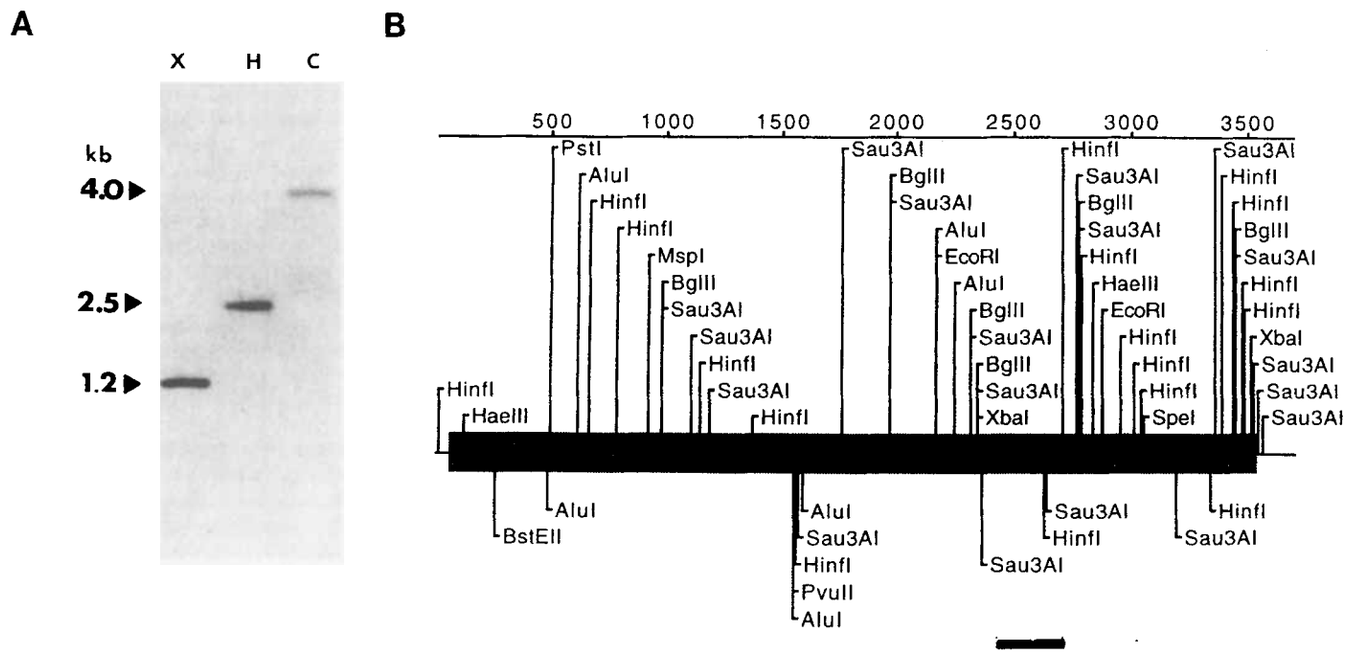

Fig. 1. Genomic Southern blot analysis and Restriction Map. **A**. Genomic DNA was cleaved with restriction enzymes, electrophoresed through agarose, capillary blotted to a nylon membrane, then hybridized with a digoxigenin-labeled probe (the initial PCR product of 285 bp). Detection by chemiluminescence was done as described in Materials and Methods. The following restriction enzymes were used: *Xba* I (X), *Hae* III (H), and *Cla* I (C). **B**. Restriction map of the full open reading frame of the calcium pump gene with the location of the probe from B shown as a bar below.

clones resembled Na/K ATPases or were PCR artifacts and the PCR primers were not recognized in the sequences.) This sequenced region was then used as a probe for Southern blots (Fig. 1) in order to clone upstream and downstream through inverse PCR methods (see following section).

Inverse polymerase chain reaction. The initial 285 bp PCR product served three purposes: to confirm that the plasma membrane Ca^{2+}-ATPase might exist in *Paramecium*; to provide a probe for Southern blots; and to provide the known sequence in order to design Inverse PCR primers. Inverse PCR uses genomic DNA that has been digested with restriction enzymes and circularized by ligation. The DNA is then amplified using primers that were synthesized in the opposite orientation compared to those used for standard PCR. In order to determine which restriction enzymes to employ, the Southern blots of genomic DNA were probed with the digoxigenin–labeled 285 bp region. Southern blot results showed three bands present: 1.2 kbp, 2.5 kbp, and 4.0 kbp in DNA cut with *Xba* I, *Hae* III, or *Cla* I, respectively (Fig. 1).

Genomic DNA fragments that were cut with *Xba* I and *Hae* III were ligated to form monomeric circles, then used as the template for Inverse PCR. The Inverse PCR primers to be used on the *Xba* I and *Hae* III digested genomic DNA (Table 1) were designed from the original amplification product sequence. Inverse PCR amplification provided the expected 1.2 kb and 2.5-kb products. Each PCR product was then directionally in-

Table 1. Inverse polymerase chain reaction primers.[a]

Primer	Sequence	RE[b]	Size[c]
Forward	CGTCCTCGAGTAAGATAATGGCAGAAGC		
	*Xho*I	*Xba*I	1.2 kb
		*Hae*III	2.5 kb
Reverse	TTCAATCGATTGTCAATGTTGTTGCAGTGG		
	*Cla*I		
Forward	ATTACTCGAGGCACATGTTGGC		
	*Xho*I	*Eco*RI	1.5 kb
Reverse	GGACTTAGGATCCAATAGTGGG		
	*Bam*HI		
Forward	GCATCAAGTAGTACTCCATCTGCTGG		
	*Sca*I	*Cla*I	2.5 kb
Reverse	GGTTGGACTCGAGGTAAG		
	*Xho*I		

[a] All primers are written in the 5′–3′ direction with internal cut sites underlined. These cut sites were used to ligate the inverse PCR product into the plasmid.

[b] RE, restriction enzyme used to cut the genomic DNA prior to inverse PCR.

[c] Length of the expected inverse PCR product.

serted into the cut KS+ pBluescript plasmid. Sequencing of each of the inserted inverse PCR products (1.2 kb and 2.5 kb) provided an overlapping region of 489 bases, thus giving a total sequence of 3.2 kb, and, predicting from the published Ca-ATPase sequences, leaving a 0.5 kb segment yet to be amplified.

Genomic DNA was cleaved with *Cla* I, amplified by inverse PCR and ligated into the pBluescript vector. Sequencing of the 2.5 kb inverse PCR product provided a sequence that included the proposed start codon and a region 75 bases upstream of that start codon. Thus, sequencing of the three inverse PCR products provided 3,500 bases including the proposed start codon and upstream region; however, there was no stop codon. The last 500 bases of the known 3′ region sequences were labeled with digoxigenin and used as a probe for another Southern blot. Genomic DNA was cleaved with *Eco R* I, ligated, amplified by inverse PCR (see Table 1 for primers), inserted into the pBluescript plasmid and sequenced. This provided both the proposed stop codon and downstream sequence.

Final PCR amplification and sequencing of the plasma membrane Ca^{2+}-ATPase. The entire open reading frame was amplified to produce the full-length plasma membrane Ca^{2+} pump PCR product for confirming sequencing. Primers were designed from the region 80 bases upstream of the start codon and the region 155 bases downstream from the stop codon. Final PCR amplifications were performed using decreased amounts of dNTP (50 μM) compared to those used initially (200 μM) in order to reduce nucleotide misincorporation from the *Taq* polymerase. The final PCR product included 3,483 bases of open reading frame for the plasma membrane Ca^{2+} pump, 66 bases upstream of the proposed start codon, and 148 bases downstream from the stop codon.

Nucleotide and amino acid sequence analysis. Using the MacVector Program, the open reading frame for the plasma membrane Ca^{2+} pump gene from *Paramecium tetraurelia* appears to begin with ATG, which is consistent with other sequences from *Paramecium* [15, 25]. The next in-frame ATG was located 378 bases downstream from the assumed start codon (Fig. 2). This ATG was not thought to be the start codon for this gene because it followed deduced amino acids that aligned with conserved amino acids present in Ca^{2+} pumps. Two examples are the aspartate (D) #92 and the asparagine (N) #103 (Fig. 2). The aspartate is conserved for all plasma membrane Ca^{2+} pumps [5, 10, 14, 33, 34, 36, 38] while the asparagine is conserved in both ER and plasma membrane Ca^{2+} pumps [5, 10, 14, 33, 34, 36, 38]. The 66 bases upstream of the assumed start codon contained two stop codons (TGA) between the next upstream ATG codon and the assumed start ATG.

The upstream region showed little resemblance to consensus sequences such as the TATA, GC, or CCAT boxes usually found in eukaryotic promoters [16]. A TATAA sequence was located 13 bases upstream from the start codon (Fig. 2), close to an expected eukaryotic initiation factor binding site, but the significance of this sequence in a highly AT rich region is questionable. Likewise, the 148 bp found 3′ to the stop codon offered little information. The 3′ noncoding region contained characteristic T "runs" (4–5 Ts) located between nucleotides 3,609–3,654 (Fig. 2) that are found in eukaryotes as the transcription termination signals [16]. However, these "Ts" were found over 100 bases past the stop codon.

The G-C content of the coding region was higher than that of the upstream and downstream flanking regions. The coding region contained 33.6% G-C, which was similar to the 35–38% G-C found in the 156G [16], 51A [24], and 51C [25] *Paramecium* surface antigen genes. The upstream and downstream

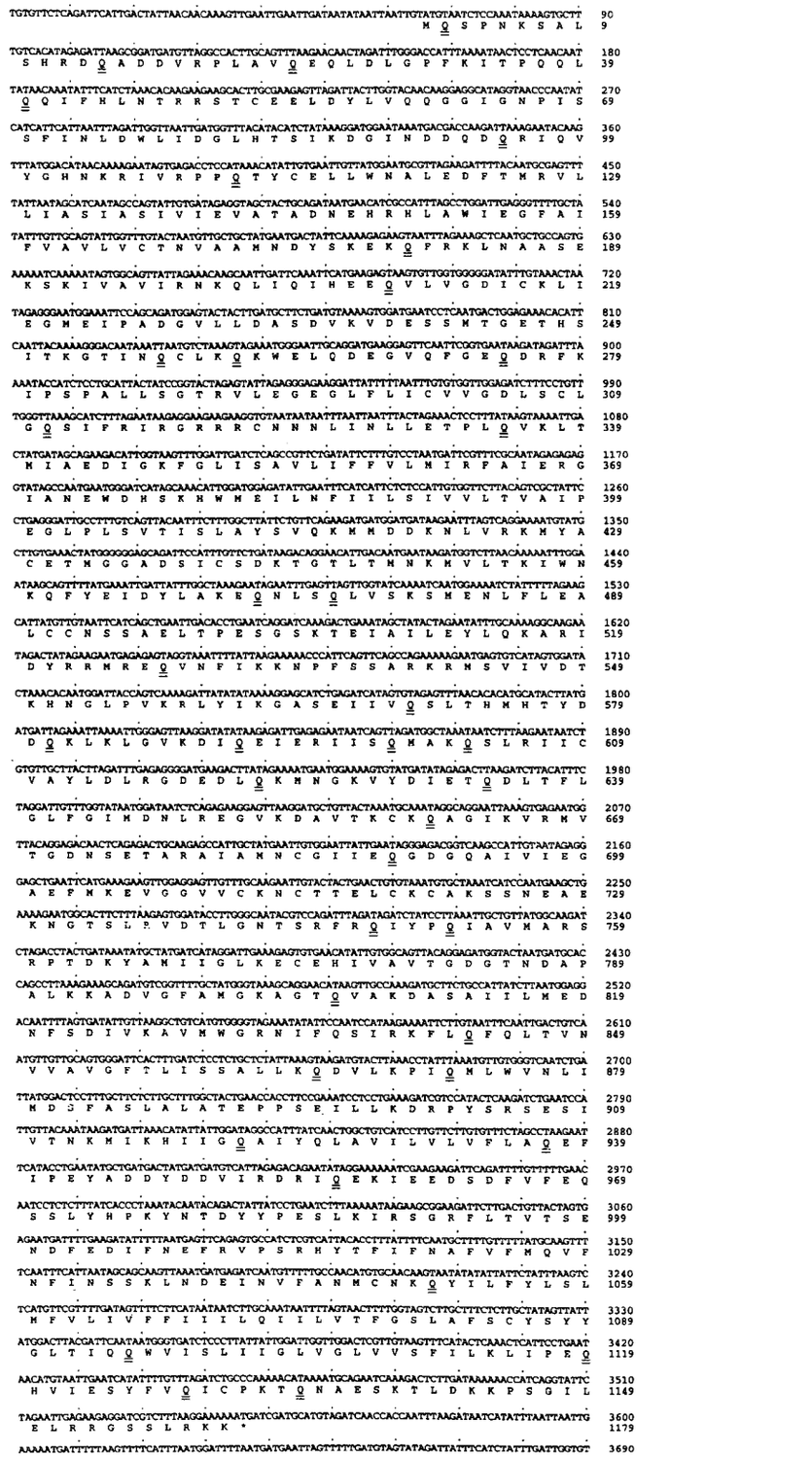

Fig. 2. The nucleotide and predicted amino acid sequence of the plasma membrane Ca^{2+}-ATPase gene from *Paramecium tetraurelia*. In *Paramecium* codon usage, UAA and UAG can encode for glutamine, designated by double underlined Q.

FIGURE 3

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ATP BINDING REGION
          560
hPMCA2    YSKGASEIV
rPMCA2    YSKGASEIV
hPMCA1    FSKGASEII
pPMCA1    FSKGASEII
ptPMCA    YIKGASEII

PMCA HINGE REGION
          778                                   809
hPMCA2    VAVTGDGTND GPALKKADVG FAMGIAGTDV AK
rPMCA2    VAVTGDGTND GPALKKADVG FAMGIAGTDV AK
hPMCA1    VAVTGDGTND GPALKKADVG FAMGIAGTDV AK
pPMCA1    VAVTGDGTND GPALKKADVG FAMGIAGTDV AK
ptPMCA    VAVTGDGTND APALKKADVG FAMGKAGTQV AK

PMCA CALCIUM TRANSPORT REGION
          872                        898
hPMCA2    QMLWVNLIMD TFASLALATE PPTETLL
rPMCA2    QMLWVNLIMD TFASLALATE PPTETLL
hPMCA1    QMLWVNLIMD TLASLALATE PPTESLL
pPMCA1    QMLWVNLIMD TLASLALATE PPTESLL
ptPMCA    QMLWVNLIMD SFASLALATE PPSEILL

PMCA ACYLPHOSPHATE INTERMEDIATE REGION
          393                                                       442
hPMCA2    VLVVAVPEGL PLAVTISLAY SVKKMMKDNN LVRHLDACET MGNATAICSD
rPMCA2    VLVVAVPEGL PLAVTISLAY SVKKMMKDNN LVRHLDACET MGNATAICSD
hPMCA1    VLVVAVPEGL PLAVTISLAY SVKKMMKDNN LVRHLDACET MGNATAICSD
pPMCA1    VLVVAVPEGL PLAVTISLAY SVKKMMKDNN LVRHLDACET MGNATAICSD
ptPMCA    VLTVAIPEGL PLSVTISLAY SVQKMMDDKN LVRKMYACET MGGADSICSD

          443      452
hPMCA2    KTGTLTTNRM
rPMCA2    KTGTLTTNRM
hPMCA1    KTGTLTMNRM
pPMCA1    KTGTLTMNRM
ptPMCA    KTGTLTMNKM

PMCA CALMODULIN BINDING DOMAIN
          1150                          1178
hPMCA2    ELRRGQILWF RGLNRIQTQI RVVKAFRSS
rPMCA2    ELRRGQILWF RGLNRIQTQI RVVKAFRSS
hPMCA1    ELRRGQILWF RGLNRIQTQI RVVNAFRSS
pPMCA1    ELRRGQILWF RGLNRIQTQI RVVNAFRSS
ptPMCA    ELRRGSSLRK K..... .....

hPMCA2=human PMCA isoform 2 (accession #Q01814)
rPMCA2=rat PMCA isoform 2 (accession #P11506)
hPMCA1=human PMCA isoform 1 (accession #P20020)
pPMCA1=pig PMCA isoform 1 (accession #P23220)
ptPMCA=Paramecium tetraurelia PMCA (accession #U05880)
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Fig. 3. GCG Pile Up comparison of *P. tetraurelia* plasma membrane Ca^{2+}-ATPase protein sequence with other known sequences from human (hPMCA isoforms 1 and 2), rabbit (rPMCA isoform 2) and pig (pPMCA isoform 1) [5, 11, 17, 33, 38]. The amino acid numbering is based on the *P. tetraurelia* sequence.

flanking regions contained 21.8% and 22.2% G-C respectively, typical of the flanking regions in other *Paramecium* genes with 15–20% G-C [24, 25, 31].

The base frequency and codon usage of the open reading frame were also examined. The order of base frequency from highest to lowest was A (34.34%), T (31.92%), G (20.26%), then C (13.32%). Interestingly, this order was the same for the dihydrofolate reductase-thymidylate synthase gene (DHFR-TS) in *Paramecium tetraurelia,* also recently cloned in our laboratory [31]. In addition to the high frequency of A and T bases in the plasma membrane Ca^{2+}-ATPase sequence from *Paramecium tetraurelia,* the codon usage frequency also revealed a high tendency for the amino acid codons to end in A or U. Of the 1,161 codons that encode the plasma membrane Ca^{2+}-ATPase in *Paramecium tetraurelia,* 72.78% end in either U or A which is slightly less than the reported range of 76–83% found in other *Paramecium* genes [22, 31], but in good agreement with the 72.4% found in the *Paramecium* DHFR-TS gene, that was recently cloned in our laboratory [15, 31].

Protein analysis of the proposed plasma membrane Ca^{2+}-ATPase. The proposed plasma membrane Ca^{2+}-ATPase protein would have 1,161 amino acids (Fig. 2), with a deduced molecular weight of 131 kDa. The first 15 homologous sequences from a search of the GCG (Genetics Computer Group, Inc., Madison, WI) database were plasma membrane Ca^{2+}-ATPases.

Table 2. Amino acid sequence comparisons of *P. tetraurelia* deduced plasma membrane Ca^{2+} pump with other plasma membrane Ca^{2+} pumps.[a]

Species	% Identity	% Similarity	Gaps	AC#
Human eryth.	43.45	65.18	25	M25874
Brain	41.21	63.75	26	P11505
Rabbit muscle	40.24	63.23	26	Q00804
rPMCA2	42.17	65.56	29	P11506
hPMCA2	41.94	64.67	28	Q01814
pPMCA1	41.31	63.65	26	P23220
hPMCA1	41.41	63.55	26	P20020
Human ER	28.54	53.18	31	P16615
Yeast	30.40	55.37	28	P13586

[a] The GCG Gap alignment program was used with a gap weight of 3.0 and a length weight of 0.10. The sequences include plasma membrane Ca^{2+} pumps from human erythrocytes [35], rabbit smooth muscle [14], rat brain [33], human isoforms 1 and 2 (hPMCA1 and 2 [11, 17, 38], pig isoform 1 (pPMCA1 [5] and rat isoform 2 (rPMCA [33]). Additionally an endoplasmic reticulum Ca^{2+}-ATPase (human intestine [18]), and a Ca^{2+}-ATPase from yeast [27] are compared.

To date, there are 16 cloned and published sequences for the plasma membrane Ca^{2+}-ATPase.

The GCG Pile Up program was used to compare the proposed sequence with four plasma membrane Ca^{2+}-ATPase sequences: rat isoform 2, human isoforms 1 and 2, and pig isoform 1 [1, 5, 17, 33, 38] (Fig. 3). The proposed *P. tetraurelia* sequence contained all the characteristic sites found in association with plasma membrane Ca^{2+}-ATPases. These sites were the ATP (FITC) and acylphosphate formation binding sites, along with the "hinge" region. The proposed sequence has a short calmodulin binding region, with only 11 of the 30 amino acids present in the mammalian sequences. Additionally, conserved amino acids methionine (M) and glutamine (Q) were present at positions #1,167–1,168, and region #981–994 were comparable to the conserved sequence KFLQFQLTVNVVAV.

The proposed protein sequence was compared to other known Ca^{2+}-ATPases through the use of the GCG Gap program to determine homology, identity and similarity for the sequences. The results showed the greatest homology and identity for the proposed plasma membrane Ca^{2+}-ATPase in *Paramecium tetraurelia* were with the plasma membrane Ca^{2+}-ATPase from human erythrocytes (Table 2), with 43.4% identity for these two sequences. The *Paramecium tetraurelia* Ca^{2+}-ATPase showed the lowest sequence homology to the endoplasmic reticulum Ca^{2+}-ATPase from human intestine (Table 2).

Northern analysis. Both poly A-selected mRNA and total RNA were run on a denaturing gel (Fig. 4A, lanes 2 and 3). Half of the gel was stained with ethidium and half was used for a Northern blot, which was probed with a PCR product from the final 900 bases of the 3′ end of the gene. Included in this probe sequence was a region thought to be conserved for Ca^{2+}-ATPases, which might play a role in transporting Ca^{2+} across the membrane. When the mRNA was probed, one band $\approx$ 3.6 kb was clearly visible (Fig. 4).

DISCUSSION

Plasma membrane Ca^{2+}-ATPase sequence analysis. A *Paramecium tetraurelia* genomic clone for a plasma membrane Ca^{2+}-ATPase has been isolated using inverse PCR. Sequence analysis shows that there is an open reading frame of 3,483 nucleotides to which there is no need to add or remove nucleotides to the sequence to maintain an open reading frame and maintain alignment with other plasma membrane Ca^{2+}-ATPase

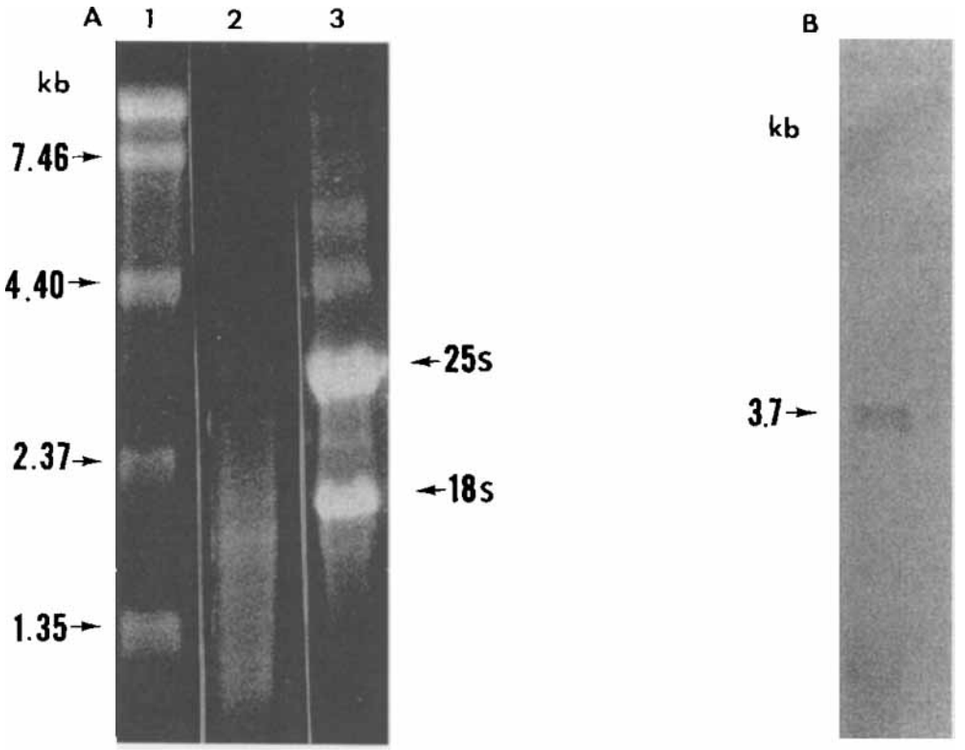

Fig. 4. Denaturing agarose gel and Northern blot analysis of *Paramecium* RNA. **A.** mRNA was isolated by using a mRNA selection kit (poly A^+ mRNA selection). Lane 1 shows the RNA markers. Lanes 2 and 3: ≈ 5 μg mRNA and ≈ 30 μg total RNA respectively. The two rRNA subunits are indicated in Lane 3. **B.** 10 μg of mRNA was probed (Lane 1) with a [^{32}P]-labeled probe, containing the final 900 bases of the plasma membrane Ca^{2+}-ATPase sequence. The arrow shows the 3.7 kb plasma membrane Ca^{2+}-ATPase message.

gene sequences. Thus, there appear to be no introns present in this genomic sequence, which is not unusual for *Paramecium.* Many of the sequenced genes of this organism (for example, the surface anitigens) contain no introns in their sequences [24, 25]. When introns are present, they are small (20–30 bp) and strictly follow the intron/exon boundary consensus sequences for eukaryotes [28]. Since there are no GT sequences followed by AG about 20–30 bp downstream, there is no evidence for introns in the *Paramecium* plasma membrane Ca^{2+}-ATPase sequence. If there are larger introns, they also maintain reading frame and fortuitously contain sequences conserved in the Ca^{2+}-ATPase genes.

The plasma membrane Ca^{2+}-ATPase gene is A–T rich (66%) which is characteristic of genes cloned in *Paramecium* [16, 24, 25, 31]. The high A–T content also is reflected in the codon usage. The 73% of the codons ending with an A or U closely agrees with the 76–83% found in other *Paramecium* genes [15, 28, 31].

The flanking regions surrounding the coding region do not appear to have consensus sequences. For example, the 66 nucleotides of the 5′ flanking region do not contain the GC or CCAT boxes usually found in the eukaryotic promoter regions [16]. There is little information about consensus sequences in the flanking regions of *Paramecium* genes, but the upstream region of this Ca-ATPase gene does have a TATAA sequence located 13 bases upstream of the start codon. While similar to the TATAAAA box sequence, it is not in the expected location. Additionally, the 3′ flanking regions do not contain any conserved sequences. However, the sequence of flanking regions further upstream could still reveal response elements.

The Southern blot analyses used for inverse PCR cloning (Fig. 1) shows hybridization that is consistent with a single gene for the plasma membrane Ca^{2+}-ATPase in the *Paramecium* genome. Different stringencies were not tested, however, and we expect that at low stringency other related sequences for the intracellular Ca^{2+}-ATPases would be evident.

Protein analysis of the plasma membrane Ca^{2+}-ATPase. The predicted protein sequence for the plasma membrane Ca^{2+}-ATPase has an associated molecular weight of 131 kD, in good agreement with the 3.7 kb transcript found in Northern blots, the 1,169–1,258 amino acids found in the plasma membrane isoforms [34] and the 128–140 kD calculated molecular weight for these isoforms [33, 34]. The calmodulin binding domain accounts for the characteristic molecular weight of plasma membrane calcium pumps, which are larger than the intracellular Ca^{2+}-ATPases that are not calmodulin regulated [3].

This sequence has the closest alignment with the plasma membrane Ca^{2+}-ATPase from human erythrocytes [11] with a 43% identity and 65% similarity (Table 2 and Fig. 3). Thus far, all of plasma membrane Ca^{2+}-ATPase genes sequenced have been from mammals, and the predicted sequence for the *P. tetraurelia* plasma membrane Ca^{2+}-ATPase gene has a 41–43% identity and 63–65% similarity with these 11 mammalian plasma membrane Ca^{2+}-ATPase genes. Conserved regions (Figs. 2, 3) include: the proline (P) at the "hinge" region within the conserved sequence VTGDGDTNDGPALKKAD found at amino acids 921–936; the acylphosphate (D) region within the sequence ICSSDKTGTLT, between amino acids 524–533; and the ATP (FITC) binding lysine (K) residue found in the KGASE sequence between amino acids 690–696.

A region that is generally found in plasma membrane Ca^{2+}-ATPase genes appears between the amino acids 1,013–1,040 (Fig. 2) and is highly conserved in *Paramecium tetraurelia.* This region has been suggested to be involved with moving the Ca^{2+} ion across the membrane [38].

Perhaps the most important differences among the *Paramecium* and mammalian plasma membrane Ca^{2+}-ATPase sequences lie in the calmodulin binding region. To date, all known

plasma membrane Ca^{2+}-ATPases have at their C terminus a calmodulin binding domain of 30 amino acids, the first 19 amino acids of which are conserved for all known isoforms [33, 39]. Enyedi et al. [7, 8] have shown with synthetic peptides for the erythrocyte plasma membrane Ca^{2+}-ATPase that amino acids 2–16 of the original 30 amino acids are sufficient to bind calmodulin. They did not examine shorter peptides [16], except the synthetic peptide made of amino acids 16–30, which did not bind calmodulin. The *Paramecium tetraurelia* Ca^{2+} ATPase gene product aligns with the mammalian sequences with no need to add or delete bases to maintain reading frames, but the *Paramecium* sequence is shorter by 19 amino acids at the C terminus. The *Paramecium* putative calmodulin binding domain has 11 amino acids, six of which are conserved among the first 19 of the mammalian binding domain. Therefore, it is possible the *Paramecium* Ca^{2+}-ATPase binding domain does, indeed, bind calmodulin.

There are at least two additional arguments in support of the fore-shortened calmodulin binding domain of *Paramecium* binding calmodulin. First, the *Paramecium* does not show the acidic regions that flank the calmodulin binding domains in mammalian sequences. The function of the acidic sites is not known but probably affects the affinity of the Ca^{2+}-ATPase for calmodulin. Enyedi and co-workers [7, 8] found in the mammalian sequences that a 28 amino acid peptide comprised of the amino acids 2–29 bound to calmodulin 40 times more tightly than the native enzyme itself. They theorized that the high affinity of the segment of the basic calmodulin binding domain was necessary to overcome the acidic domains that flank the calmodulin binding domain in the intact calmodulin binding region [7, 8, 10, 11]. The sequence for the plasma membrane Ca^{2+}-ATPase in *Paramecium tetraurelia* has no acidic regions flanking the calmodulin binding domain (Fig. 3). Perhaps the lack of acidic regions compensates for the shortened calmodulin binding site, if indeed the small size of the site reduces its intrinsic affinity for calmodulin.

Other evidence involving the N-terminus of the calmodulin binding domain, which is thought to be involved in binding and regulating the Ca^{2+} affinity for the plasma membrane Ca^{2+}-ATPase suggests the C terminus of the plasma membrane Ca^{2+}-ATPase can bind and be regulated by calmodulin [7]. Enyedi et al. [8, 13] have demonstrated that the effects of various size synthetic peptides on the $K_{1/2}$ for Ca^{2+} are associated with the net charge present on the peptide and not the presence or absence of the C-terminal end of the calmodulin binding domain. They found that a longer synthetic peptide with amino acids 2–21 (of the 30 amino acids of the calmodulin binding region) has a +5 net charge and gives the same binding results as the longer peptide (amino acids 2–29) which also has a +5 net charge. The sequence for the *Paramecium tetraurelia* gene's calmodulin binding domain likewise has a +5 net charge. The calmodulin binding regions from four different isozymes of the plasma membrane Ca^{2+}-ATPase show four basic residues present in the rat 1 isozyme from rat brain [33], five in the human erythrocyte [8], six in human teratoma [3], and seven basic residues in the rat brain 2 isozyme [33]. Thus, the proposed calmodulin binding domain for the sequence from *Paramecium tetraurelia* with five basic residues may have sufficient basic residues present to bind calmodulin and activate the Ca^{2+} pump.

Significance. *Paramecium* has long been recognized as a useful organism for the study of excitable membrane properties, including its calcium action potential [37], yet it has never been evident how the cells remove or sequester the calcium that enters through the voltage-gated calcium channels of the cilia. More recently it has become apparent that a calcium pump current plays a role in chemical sensing signal transduction in *Paramecium* [40–42]. The identification of a plasma membrane Ca^{2+} pump proved to be problematic until it was recently recognized this important enzyme is localized to the cell body membrane and not to ciliary membrane where some if not all of the calcium channels reside [6, 41, 42]. Once it became evident the enzyme had characteristics of the plasma membrane Ca^{2+} pumps across phyla [40], it was feasible to clone the gene through inverse PCR with primers based on conserved sequences. Therefore, the cloning of the plasma membrane calcium pump gene from *Paramecium* opens up possibilities for the manipulation and study of the role of this enzyme in many cellular functions. Since the cloned gene has such high similarity to other plasma membrane calcium pump genes, it should be possible to manipulate and study its function in *Paramecium* where small changes in calcium homeostasis are amplified by the dramatic swimming behavior changes they produce, and extrapolate the results to other cell systems that are not as amenable to perturbations in calcium pump function or show dramatic changes. The understanding of pumps and transporters that move and sequester Ca^{2+} promote our understanding of how intracellular Ca^{2+} is regulated and the regulation of Ca^{2+}_i is essential for normal cellular functions.

Note Added in Proof: Since the writing of this manuscript, a sequence for the plasma membrane Ca^{2+}-ATPase gene from *Entamoeba histolytica* (accession #U20321) has been submitted to GCG. This gene has a very short calmodulin binding domain of 16 amino acids, very similar to that of the 11 amino acid domain of *P. tertraurelia*. *P. tetraurelia* plasma membrane Ca^{2+}-ATPase gene is 61.3% similar and 37.7% identical to this gene.

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Received 5-15-95, 12-5-95, 11-14-96; accepted 2-10-97