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P-TYPE ATPASES IN TETRAHYMENA THERMOPHILA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Shusheng Wang, M.S.

*****

The Ohio State University

1996

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ABSTRACT

The P-type ATPase gene family of ion pumps is widely distributed in fungi, protozoa, plants, and animals. However, in spite of detailed sequence analysis which shows that these ion pumps have highly diverged primary structures, except at a limited number of amino acid residues important for ATP-hydrolysis, functional characterization of many pumps is still lacking. Using degenerate oligonucleotides corresponding to the phosphorylation and CIRATP-binding sites, we applied a PCR technique to isolate genes encoding ion pumps in *Tetrahymena*, as a basis for evolutionary and physiological studies. In total, 12 distinct P-type ATPase genes were identified and classified into 3 families, related closely by sequence. Family 1 (at least 7 homologous genes) encodes ion pumps similar to animal Na\(^+\)/K\(^+\) (H\(^+\)/K\(^+\)) ATPases, family 2 (1 gene) resembles SERCA ATPase gene, and family 3 gene products are functionally unclassified but share significant homology with Plasmodium ATPase1 and an ATPase from C. elegans.

A Southern blot analysis revealed the existence of the distinct P-type ATPase genes in the *Tetrahymena* genome; the family 1 contains at least 10 genes, while the genes in the other two families do not seem to possess such multiplicity. A Southern blot analysis following pulse-field gel electrophoresis showed that all these genes are located in *Tetrahymena* macronuclei, with members of family 1 widely distributed on at least 4 different macronuclear chromosomes. The more recent duplications created two genes closely linked on the same macronuclear chromosomes, whereas the genes derived from the older duplications have drifted to the different chromosomes. Phylogenetic analysis suggests that *Tetrahymena* family 1 branched off from the phylogenetic lineage of the Na\(^+\)/K\(^+\) (H\(^+\)/K\(^+\)) ATPases, possibly before the separation of the Na\(^+\)/K\(^+\) and the H\(^+\)/K\(^+\)-
ATPase. It is interesting to note that the family 1 evolved in this single-celled organism with a high frequency of gene duplication resulting in at least 10 isoforms.

A Northern blot analysis under various culture conditions [i.e., at different temperatures (25°C, 30°C, 37°C and 42°C), in high salt solution (0.2 M NaCl) and under starvation (10 mM Tris-HCl, pH 7.5)] constantly revealed a single size of transcript (~3.6 kb) for TPA2 (Na⁺/K⁺- or H⁺/K⁺-ATPase), TPA8 (SERCA-ATPase), and TPA9-10 (two of the protozoan ATPases). A high concentration of NaCl did not result in apparent changes in the mRNA levels. However, a drastic increase in the level of TPA2- and TPA10-expression was seen at 37°C. Starvation down-regulates the expression of the TPA2, TPA8, TPA10 genes as well as the histone H4-I gene, but up-regulates TPA9 expression. The TPA9 gene product with a molecular weight of 130 kDa was immunolocalized at the oral apparatus, a place undergoing phagocytosis for particulate food uptake, whereas TPA10 was immunolocalized to mucocysts, secretory granules in the cortex of cell bodies.

A comparison of the full-length sequences suggests that TPA2 is most similar to animal Na⁺/K⁺-ATPase family. With the average 40% of identity, TPA2 is the most divergent member in Na⁺/K⁺-ATPase family. The divergence is even larger than that (60%) between Na⁺/K⁺-ATPase and H⁺/K⁺-ATPase. The analysis reveals three distinct features for TPA2: 1) The ouabain binding domain between the first and the second transmembrane domains is five amino acid residues shorter than those of Na⁺/K⁺-ATPases. 2) The extracellular loop between transmembrane domain H7 and H8 is 55 amino acids longer than the corresponding region in animal Na⁺/K⁺-ATPases. This region has been shown to be the assembling site with a small glycoprotein β subunit. 3) Several glutamates as well as asparagine and threonine residues within transmembrane domains 4, 5, 6 and 8, are conserved. These results are consistent the current idea that these charged amino acids are important for cation transport.
To my father and my family
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PUBLICATIONS

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**FIELD OF STUDY**

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CHAPTER 1

INTRODUCTION

1.1 The general principle of ion transport

Since ions are hydrated in solution and do not readily traverse the hydrophobic lipid bilayer of membranes, translocation across the plasma membrane occurs via transport proteins. The ion flux across membranes is dependent on the thermodynamic gradient ($\Delta \mu$). $\Delta \mu$ consists of two components, the electrical gradient or membrane potential and the chemical gradient. Transport of ions down the $\Delta \mu$ is passive, whereas transport against the gradient is active. The transport proteins that mediate ion translocation can be generally categorized as pumps, carriers, and channels. Pumps directly use energy provided by ATP hydrolysis to create electrochemical gradients, whereas carriers couple uphill transport of one solute to the downhill movement of another, either in the same (symporter) or opposite (antiporter) direction. Channels mediate passive transport, i.e. movement down a free energy gradient. The activities of these membrane proteins provide a basis for regulation of ion homeostasis in all living cells. In this chapter, I will give a more detailed introduction to ion pumps.
1.2 Three types of ion pumps

By the above definition, ion pumps are enzymes that can directly couple the ATP hydrolysis to ion translocation against their gradients. In this sense, ion pumps are also called ion-motive ATPases. The ion-motive ATPases discovered to date can be grouped into three classes: F-type ATPases, V-type ATPases and P-type ATPases (Pedersen and Carafoli, 1987).

F-type ATPases are located in bacterial plasma membranes, inner mitochondrial membranes, and thylakoid membrane of chloroplasts. The in vivo function of this type of enzymes is to couple the downhill movement of protons to the synthesis of ATP from ADP and inorganic phosphate. Thus the F-type ATPases are ATP synthases. However, under suitable conditions ATP synthases can undergo a reverse direction in which the enzymes can hydrolyze ATP to move protons against the electrochemical gradient (Amzel and Pedersen, 1983). The stoichiometry of $\text{H}^+/\text{ATP}$ is three. F-type ATPases consist of a large, multienzyme (500 kDa) made up of two major complexes, $F_1$ and $F_0$. $F_1$ is water-soluble catalytic complex made up of five subunits ($\alpha - \epsilon$) with the arrangement of $\alpha_3\beta_3\gamma\delta\epsilon$. The catalytic site is located on $\beta$ subunit. The slightly larger $\alpha$ subunit contains nucleotide binding sites and participates in catalysis as a regulatory subunit. $F_1$ can be readily removed from the membrane in catalytic active form by treatment with EDTA or application of mechanical force. $F_0$ is made of at least three integral membrane proteins ($a - c$) with the arrangement of $ab_2c_{6-12}$ and the 8 kDa hydrophobic subunit $c$ assembles to form the proton channel. The inhibitor DCCD binds
to a negative charged amino acid residue within the channel pore and blocks proton translocation.

V-type ATPases are primary located in intracellular membranes, particularly the lysosomal and vacuolar membrane of eukaryotic cells. Using the mechanism analogous to F-type ATPases, V-type ATPases acidify intracellular compartments such as vacuoles, endosomes, lysosomes, Golgi, chromaffin granules and coated vesicles (Harvey, 1992). V-type ATPases share a number of features in common with F-type ATPases. They are large, multimeric enzymes (nearly 500 kDa) composed of an integral membrane moiety and a hydrophilic catalytic complex. Like $F_1$, the hydrophilic complex contains 5 subunits (A-E) with a stoichiometric ratio of $A_3B_3CDE$. Unlike F-type ATPases, the membrane associated $V_0$ domain is composed of only two subunits (a and c) with a stoichiometric ratio of $(acg_{6-12})$. They are insensitive to azide and are inhibited by nitrate, N-ethylmaleimide (NEM), and two novel classes of inhibitors, bafilomycin and concanamycin (Nelson and Taiz, 1989).

P-type ATPases are broadly distributed. They are responsible for the active transport of a variety of cations across membranes. Translocation of cations against their electrochemical gradients is achieved by utilizing the energy of ATP hydrolysis. Formation of a covalent acylphosphate enzyme intermediate during the cycle of ATP hydrolysis and cation translocation gives them the “P” designation, thus distinguishes them from the F-type and V-type ATPases.

P-type ATPases are located in the plasma membrane or in the membrane of specialized organelles such as the endoplasmic reticulum and sarcoplasmic reticulum and
are primarily responsible for the maintenance of the ionic composition of the intracellular milieu, which is essential to the function of many enzymatic reactions in the cytoplasm, i.e. a high potassium concentration (100 mM), a low sodium concentration (1-10 mM), a pH around 7.0-7.5, and a very low concentration of calcium and other divalent cations such as copper, zinc etc. Currently, new evidence suggests that a new family of P-type ATPases is also involved in the asymmetric distribution of aminophospholipids (Tang et al., 1996).

In contrast to F-type and V-type ATPases, P-type ATPases have a simpler structure. All P-type ATPases include a main polypeptide with the molecular weight of 70-130 kDa. This polypeptide is usually called α subunit or catalytic subunit because it possesses the elements directly required for ATP hydrolysis and ion translocation. Several P-type ATPases such as Na⁺/K⁺-ATPases and H⁺/K⁺-ATPases are composed of two subunits, the catalytic α subunit and the β subunit. The β subunit is a small glycoprotein (30-50 kDa) which assembles with the α subunit for functional enzymes. The K⁺-activated P-type ATPase in E. coli is composed of three subunits named as A, B and C. The B subunit is catalytic subunit, homologous to the α subunit of eukaryotic P-type ATPases. Another feature that distinguishes the P-type ATPase from the F-type and V-type ATPases is their sensitivity to vanadate.

1.3 The diversity of P-type ATPases

Up to date, more than 60 P-type ATPases have been found from a variety of organisms. As for many other types of enzymes, the P-type ATPase superfamily had
been defined initially by its biochemical and pharmacological characteristic, i.e. an ATP hydrolytic activity dependent on the presence of certain ions and inhibited by vanadate. However, over the last ten years, the sequences of several proteins presenting these functional characteristics have been determined. Comparison of these sequences has revealed a highly conserved domain that is the sequence DKTGTLT involved in the formation of acylphosphorylated intermediate. A new definition of the P-type ATPase has emerged, based on the presence of this highly conserved domain. Newly identified sequences that include this characteristic sequence are generally considered as P-type ATPases. By applying the degenerated oligonucleotides corresponding to these conserved sequence motifs to explore the genome of a variety of organisms, the number of new members has been grown at a very fast pace over the last few years. Expansion of P-type ATPase superfamily has been highlighted with the appearance of new P-type ATPase families and the appearance of new isoforms for the known ATPase families. In the following part, I will review the major families of P-type ATPases.

1.3.1 The Na⁺/K⁺-ATPase family

The Na⁺/K⁺-ATPase is an integral membrane protein found in the cells of all higher animals and is responsible for translocating sodium and potassium ions across the cell membrane by utilizing ATP as the energy source. For three sodium ions pumped out of the cell, two potassium ions are pumped in. This transport creates both a chemical and an electrical gradient across the cell membrane. The electrical gradient is essential for maintaining the resting potential of cells and for the excitable activity of muscle and
nerve tissue. The sodium gradient is used to drive numerous transport processes, including translocation of glucose, amino acids and other nutrients into cells via a family of Na\(^{+}\)-coupled symporters, including extrusion of certain ions such as H\(^{+}\) and Ca\(^{2+}\) out of cells via a family of Na\(^{+}\)-coupled antiporters, e.g. Na\(^{+}\)/H\(^{+}\) exchanger and Na\(^{+}\)/Ca\(^{2+}\) exchanger. The sodium gradient is also important for cell volume regulation. For instance, when a cell needs to gain volume after shrinkage due to exposure to a hypertonic solution, it can rapidly import ions via the Na\(^{+}\)/K\(^{+}\)/Cl\(^{-}\) cotransporter that can use the large inwardly directed Na\(^{+}\) gradient to drive K\(^{+}\) and Cl\(^{-}\) ions into the cell. The import of these ions is followed by osmotic flow of water, allowing a rapid control of cell volume in a hypertonic environment. When a cell needs to lose volume after swelling due to exposure to a hypotonic solution, the situation is different and the outwardly directed K\(^{+}\) gradient becomes the major driving force. The opening of the volume-activated K\(^{+}\) channels will allow K\(^{+}\) ions flow out of the cell and hyperpolarize the cell membrane. In the presence of anion conductance, this will result in a net loss of K\(^{+}\) and an anion, lead to a decrease of the cell volume. Physiologically, Na\(^{+}\)/K\(^{+}\)-ATPases present in organs such as the intestines and the kidney regulate fluid reabsorption and electrolyte movement by establishing an ionic gradient across epithelial membranes. It is estimated that approximately 23% of the ATP consumed in humans at rest is utilized by sodium pumps.

A widely used model for the reaction mechanism of Na\(^{+}\)/K\(^{+}\)-ATPase is referred to as the Albers-Post scheme. The basic idea of the model is that the Na\(^{+}\)/K\(^{+}\)-ATPase can exist under two distinct conformations (E1 and E2). The equilibrium towards one or the
other conformation can be modified by the environment, i.e. the concentration of ATP, Na\(^+\), and K\(^+\). In addition, certain inhibitors bind preferentially to one conformation and tend to fix the enzymes in this conformation. When the enzyme is in the conformation such that cation-binding sites are available from the intracellular side, ATP, Mg\(^{2+}\) and Na\(^+\) bind to the enzyme intracellularly. This conformation (E1) has high affinity to Na\(^+\) and low affinity to K\(^+\). The enzyme is autophosphorylated and the bound Na\(^+\) is occluded. Subsequently, ADP is released intracellularly and followed by a spontaneous change in conformation. The E2 conformation has the cation binding sites exposed to the extracellular side with the high affinity to K\(^+\) and low affinity to Na\(^+\). This state is apparently the conformation to which ouabain can bind. As a result, Na\(^+\) is released and K\(^+\) binds extracellularly. Upon binding of K\(^+\), the enzyme is rapidly dephosphated and K\(^+\) is occluded. The release of K\(^+\) to the cell interior is facilitated by intracellular ATP. ATP is then bound to a low affinity site and allows a rapid change to the E1

Figure 1.1  A basic Albers-Post scheme
conformation. The basic Albers-Post scheme describing the reaction cycle is shown as above (modified from Repke, 1986).

Na⁺/K⁺-ATPase is composed of two subunits in equimolar ratios. These are the α subunit with a molecular weight of 130 kDa and the smaller glycosylated β subunit with protein portion accounting for 35 kDa of the overall molecular mass of 55 kDa. The α subunit contains the ATP binding site, the phosphorylation site and the site for the binding of cations and cardiac glycosides, thus it is referred to as the catalytic subunit. The functional roles for the β subunit are generally considered in the two aspects: 1) The β subunit has a major role in the biosynthetic process of the Na⁺/K⁺-ATPase. The β subunit is required to assemble with the α subunit in endoplasmic reticulum and the correct assembly is the prerequisite for the enzyme to exit ER and to be transported to the plasma membrane. 2) The β subunit may also be an essential part for the functional integrity of enzymes. This is supported by the fact that a function could never be reconstituted with the α subunit alone. The direct evidence provided by Kawamura and Nagano (1984), who showed that reduction of β subunit disulfide bonds was correlated with the loss of activity. Current studies show that the different isoforms of β subunit affect the affinity of external K⁺ for Na⁺/K⁺-pump current activation.

There are three isoforms for both α and β subunits (Sweedner, 1989). The expression of these isoforms exhibits tissue-specificity. The α₁ isoform occurs in most tissues, while the α₂ isoforms is predominantly in skeletal muscle and is also detected in the brain and the heart. The α₃ isoform is restricted to neural and cardiac tissue. The β₁ and β₂ are the predominant isoforms in mammalian cells where they are ubiquitously
expressed and the $\beta_2$ appears mostly in neural tissue. The $\beta_3$ isoform was only identified in *Xenopus*.

### 1.3.2 H⁺/K⁺-ATPases

The distribution of the H⁺/K⁺-ATPases is restricted to the specialized parietal cells in the gastric glands. In resting state, the enzymes are sequestered in the tubulovesicles underneath the apical cell surface of parietal cells and the H⁺/K⁺-ATPase is inactive due to the lack of K⁺ access to interior of the vesicles. If K⁺ access is provided exogenously (i.e. by adding potassium ionophores such as valinomycin or nigericin), the proton pump can be activated even in the vesicles. With food stimulation, the vesicles fuse with the apical surface of the parietal cells; therefore, the enzymes are translocated to the apical surface and result in activation of the proton pump (Hersey and Sachs, 1995). This enzyme has the remarkable ability to transport H⁺ ions into the lumen of stomach. The concentration of HCl in the lumen of stomach can reach to 160 mM. The H⁺ ions are exchanged against an equal number of K⁺ ions with a stoichiometry of $2\text{H}^+/2\text{K}^+/1\text{ATP}$; thus, the activity of the H⁺/K⁺-ATPase is electroneutral, with no net voltage being generated across the membrane by this enzyme. The reaction mechanism of the H⁺/K⁺-ATPase is similar to that of the Albers-Post cycle of the Na⁺/K⁺-ATPase.

The H⁺/K⁺-ATPase is not inhibited by ouabain, a selective inhibitor of the Na⁺/K⁺-ATPase, but is sensitive to two types of inhibitors: the substituted benzimidazole derivatives such as omeprazole, lanzoprazole and pantoprazole; the substituted pyridyl imidazole such as the Schering compound SCH 28080 (Sachs et al., 1992). Those
inhibitors effectively inhibit H+/K+-ATPases with no effect on Na+/K+-ATPases, thus are widely used for clinical treatment of acid related diseases of the gastrointestinal tract. The former inhibits the H+/K+-ATPase in the acid-dependent manner. For example, when omeprazole is exposed to a low pH, it is converted to a reactive sulfenamide that can form a covalent bond with exposed cysteines, therefore inhibits the H+/K+-ATPase irreversibly. The latter exerts their inhibitory activity by competing with K+ for the K+ binding sites on the luminal side. Both types of inhibitors have in common that their sensitivity is enhanced by the acidic environment.

Although the ion specificity and subcellular localization of the H+/K+-ATPases are different from those of the Na+/K+-ATPases, the H+/K+-ATPases and the Na+/K+-ATPases are considered to be included into the same family based on the fact: 1) Both have the similar subunit composition with the 100 kDa catalytic α subunit and a glycosylated β subunit. The apparent molecular weight of the β subunit on SDS-PAGE is highly variable, depending on degree of glycosylation, but deglycosylated core protein is about the same size (about 34 kDa). 2) The amino acid sequences between them share a high degree of homology. The identity between the gastric H+/K+-ATPase and Na+/K+-ATPase α subunit is 60 - 65%. Similarly, the identity between the H+/K+-ATPase and Na+/K+-ATPase β subunits is 30 - 40%, values close to the identities between the β1 and β2 or β3 isoforms of the Na+/K+-ATPases (35 - 40%). 3) Hydrophobicity plots obtained from these primary structures predict that both share a similar membrane topology. 4) Currently the functional studies suggest that sodium pump is also permeable to protons in certain conditions (Wang and Horisberger, 1995).
1.3.3 Ca\(^{2+}\)-ATPases

It is essential for all types of cells to maintain a low concentration of calcium (submicromolar) in the cytoplasm during steady state and after excitation. In general, there are three mechanisms that are responsible for removing calcium from the cytoplasm: two P-type Ca\(^{2+}\)-ATPases and the Na\(^+\)/Ca\(^{2+}\) exchanger. The Na\(^+\)/Ca\(^{2+}\) exchanger, as I mentioned in the previous part, relies on the Na\(^+\) gradient to extrude Ca\(^{2+}\) out of cells. It is present in large amounts in excitable cells such as heart cells and neurons. Since it has a low affinity for Ca\(^{2+}\), it only activates when the intracellular Ca\(^{2+}\) level rises to a substantially high level. The Ca\(^{2+}\) pumps have a higher affinity for Ca\(^{2+}\). One of them is located in the plasma membrane and another in the endoplasmic reticulum or sarcoplasmic reticulum. They appear to be responsible for maintaining the low resting level of Ca\(^{2+}\) in unstimulated cells. In the following part, I will give them a more detailed description.

1) SERCA-ATPases

The SERCA-ATPases have been characterized and sequenced from a variety of organisms including yeast, fungi, protozoa, plants and animals. They pump Ca\(^{2+}\) from the cytosol into intracellular stores. In skeletal and cardiac muscle cells, the intracellular store for Ca\(^{2+}\) is the sarcoplasmic reticulum, a system of interconnecting tubules and cisternae wrapped around each myofibril. In non-muscle cells, the intracellular Ca\(^{2+}\) store is the endoplasmic reticulum. The SR Ca\(^{2+}\)-ATPase is the major protein in the SR membrane and it accounts for nearly 50% of the membrane dry weight. The high density
of SR Ca\(^{2+}\)-ATPase in the SR membrane allows Ca\(^{2+}\) be rapidly transported back to the SR lumen after muscle contraction. The Ca\(^{2+}\) removing process is coupled with muscle relaxation.

The SERCA-ATPase is composed of a single peptide of 110 kDa. It can be regulated by a small peptide of 52 amino acids. When this peptide is phosphorylated, it binds to a specific site on the SERCA and inhibits its activity. The SERCA-ATPase is inhibited by a plant toxin, thapsigargin. The affinity of thapsigargin is very high (K\(_d\) < 1 nM) for SERCA, while it has no effect on the plasma membrane calcium ATPase and on the other P-type ATPases.

The SERCA-ATPase is encoded by multiple isoforms: SERCA1, SERCA2a, SERCA2b and SERCA3. The SERCA1 isoform is predominant in fast-twitch skeletal muscle. Two forms of SERCA2 occur as a result of alternative splicing. SERCA2a isoform is predominant in slow-twitch cardiac muscle and SERCA2b isoform is dominant in smooth muscle and non-muscle cells. The SERCA3 isoform appears to be widely distributed in a variety of tissues.

2) PMCA-ATPases

The plasma membrane Ca\(^{2+}\)-ATPase is the system that extrudes Ca\(^{2+}\) out of eukaryotic cells. It is involved in the global and long term calcium balance of the cell. The most distinct feature of the PM Ca\(^{2+}\)-ATPase is its stimulation by Ca\(^{2+}\)/calmodulin (Carafoli, 1992). Four genes coding for isoforms of PMCA (PMCA1 to 4) have been cloned to date and additional isoforms are produced by alternative splicing (Carafoli, 1994). PMCA1 is widely distributed while the other isoforms are found mostly in brain.
The deduced amino acid sequences of PM Ca\textsuperscript{2+}-ATPase isoforms shares only 25% identity with those of SERCA-ATPases. A striking difference between the primary sequence of PMCA and SERCA is at the C-terminus, where the PM Ca\textsuperscript{2+}-ATPase carries an extra 150 amino acids, including two possible binding domains for calmodulin (Vorherr, 1990). This also results in a bigger molecular weight (135 kDa vs. 110 kDa) for PMCA. Proteolytic cleavage of this region of the PM Ca\textsuperscript{2+}-ATPase results in a loss of calmodulin sensitivity, making the molecule fully active in the absence of calmodulin. Synthetic peptides that correspond to the calmodulin binding domains of the PM Ca\textsuperscript{2+}-ATPase inhibit the activity of this truncated ATPase (Enyedi et al., 1989; James et al., 1989). The results suggest the existence of the auto-inhibitory domains within the C-terminal region that suppress the activity of the PM Ca\textsuperscript{2+}-ATPase. When calmodulin is activated by calcium, it binds to a 30 amino acid segment at the C-terminus. This prevents the interaction of the C-terminal region with the rest of the protein and removes the auto-inhibitory action. By this mechanism, binding of calmodulin increases the affinity for intracellular Ca\textsuperscript{2+} by a factor of about 20 and also increases the Ca\textsuperscript{2+} transport activity.

1.3.4 H\textsuperscript{+}-ATPases

Plants and fungi do not have Na\textsuperscript{+}/K\textsuperscript{+}-ATPases. The plasma membrane H\textsuperscript{+}-ATPase acts as a primary ion pump to maintain the ion homeostasis in plant and fungal cells. The proton gradient created by this enzyme provides a major driving force for transport of many solutes (ions and metabolites) into and out of the cell.
The mechanism responsible for maintaining a high concentration of potassium inside the plant cell is totally different from that in animal cells. Depending on the K⁺ concentration in the soil, K⁺ import occurs via two different transport systems. The first is a low-affinity transport system cloned and characterized as a K⁺ channel, which allows K⁺ to enter the cell when the external K⁺ concentration is higher than 1 mM (Schachtman et al., 1992; Sentenac et al., 1992). The second is a high-affinity K⁺ transport system cloned and characterized as a H⁺/K⁺ symporter. It permits plants to grow in soil in which the K⁺ concentration is as low as 27 nM while maintaining the intracellular K⁺ concentration at about 100 mM (Schachtman and Schroeder, 1994). The sodium extrusion is presumably due to the activity of a Na⁺/H⁺ antiporter. The uptake of sugar, amino acids and NO₃⁻ also relies on the proton gradient. A set of symporters for nutrient uptake has been cloned and characterized by heterologous expression in yeast.

The plant H⁺-ATPases are encoded by a multigene family. At least 7 genes in tomato and 10 genes in Arabidopsis have been reported (Ewing and Bennett, 1994; Harper et al., 1994). The expression of these genes is apparently dependent upon the cell type, developmental stage and environmental stimuli. For example, the expression of Arabidopsis thaliana AHA9 (Houlne and Boutry, 1994) and AHA10 (Harper et al., 1994) is detected only in anthers and developing seeds, respectively. AHA3 is expressed uniquely in phloem tissue, but only in the fully differentiated state (DeWitt, N.D. et al., 1991). PMA1 is also expressed in the phloem, but only in the fruit. PMA1 expression is affected by environmental conditions. Under normal growth conditions, the expression of PMA1 is often undetectable, but increases dramatically in guard cells when plantlets are
grown in a liquid medium or when leaves from soil-grown plants are immersed for a few hours in a nutritive solution or in water (Michelet et al., 1994).

The proton ATPase is composed of a single polypeptide with a molecular mass of 100 kDa. It transports one proton per molecule of ATP hydrolyzed and has a pH optimum of about 6.6 and a $K_m$ for MgATP of 0.3 to 1.4 mM. Potentials of about -150 mV and a pH difference of two units is routinely observed across plant plasma membranes. Its specific activity in purified plasma membranes is usually of the order of 1-2 μmol Pi min$^{-1}$ mg$^{-1}$ protein, and it is inhibited by vanadate, dicyclohexylcarbodiimide, diethylstilbestrol, and erythrosin B, but not by NaN$_3$ or oligomycin (two inhibitors of the mitochondrial and chloroplastic ATPases), nor by nitrate (an inhibitor of the vacuole membrane ATPase) or molybdate (an inhibitor of nonspecific phosphatases).

The membrane topology of the plant H$^+$-ATPase is similar to other P-type ATPases with 10 transmembrane domains. Both the N-terminal and C-terminal regions of the protein are on the cytoplasmic side of the membrane. An autoinhibitory region is present at the C-terminus (Palmgren et al., 1991). In the yeast H$^+$-ATPase, the C-terminal region mediates in vivo regulation in response to growth on Glc. This so called “Glc effect” increases the enzyme activity by several folds. It is suggested that the increased ATPase activity results from the phosphorylation of one or two residues in the C-terminal region by the certain membrane associated protein kinase. A calmodulin-dependent protein kinase was shown to activate the H$^+$-ATPase. Fusicosin also stimulates H$^+$-ATPase activity, possibly via the C-terminal inhibitory domain.
In addition to the P-type ATPases in eukaryotic organisms, a family of P-type ATPases has been characterized in prokaryotic organisms. The members of this family are involved in translocating a variety of cations including $K^+$, $Mg^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Zn^{2+}$, etc. In this part, I will briefly review four ATPases from this family.

1) $K^+$-ATPase in *Escherichia coli*

As I mentioned in the previous part, this ATPase consists of three integral proteins KdpA, B and C that are encoded by three genes in kdpABC operon. KdpB is a subunit homologous to the eukaryotic P-type ATPases (Hesse et al., 1984). KdpA contains 12 transmembrane segments and its function was suggested to involve the binding of potassium. KdpC is a smaller protein with a single transmembrane segment located near the N-terminus. Kdp ATPase has high affinity for potassium, thus allowing *E. coli* to grow in a low potassium medium. The mutant deficient in Kdp ATPase is not lethal but requires a high concentration of potassium in the medium. The source of energy for Kdp activity has been identified as ATP. Reconstitution experiment demonstrated that Kdp protein can take up potassium into the right-side out vesicles without requiring other ions.

2) $Mg^{2+}$-ATPase in *Salmonella typhimurium*

Magnesium is an important cofactor for many intracellular enzymatic reactions and is one of the most abundant intracellular cations in eukaryotic and prokaryotic organisms. Although the mechanism for $Mg^{2+}$ ion homeostasis is not known in eukaryotic organisms, the magnesium transport systems in *Salmonella typhimurium* have been well studied (Snively et al., 1991a). *Salmonella typhymurium* possesses three
magnesium transport systems, CorA, MgtA and MgtB. Triple mutants deficient in all three magnesium transport systems grow only in a medium containing a very high concentration of magnesium. The proteins encoded by MgtA and MgtB belong to the P-type ATPase family (Snavely et al., 1991b; Maguire, 1992). Since the MgtA and MgtB systems are only expressed in a medium with low concentration of magnesium, the ATPase activities of the MgtA and MgtB may involve in magnesium uptake. The sequences of these two ATPases are more closely related to the eukaryotic P-type ATPases than to the prokaryotic ATPases.

3) Cd\(^{2+}\)-ATPase in Staphylococcus aureus

This ATPase was found in certain strains of Staphylococcus aureus which usually carry a plasmid conferring cadmium resistance. Actually two genes (cadA and cadB) were found to be involved in detoxification of cadmium when cells were exposed to a high cadmium environment. The soluble protein encoded by cadB protects cells by binding cadmium. The membrane protein encoded by cadA is homologous to the P-type ATPases in eukaryotic organisms. It protects cells by extruding cadmium out of cells with consumption of ATP.

4) Copper ATPases

Recently, four genes encoding putative copper pumping ATPases have been cloned from widely different sources: two genes from Enterococcus hirae that are involved in copper metabolism and two human genes that are defective in the copper related Wilson and Menkes disease. We include two human genes here just for the
convenience of discussion since they share a high degree of homology to bacterial ATPases.

In Enterococcus hirae, an operon involved in copper homeostasis has been identified. It contains at least five genes in the order: copX, Y, Z, A and B. CopX, Y and Z are polar proteins and are probably involved in the regulation of the operon. CopA and cop B encode P-type ATPases of 727 and 745 amino acids, respectively. Wild-type E. hirae can tolerate up to 6 mM CuSO₄ in the growth medium. Cells defective in copB, or copA and copB lose their high level copper resistance. In contrast, disruption copA alone has no significant effect on the copper resistance. However copA-defective cells cease to grow after two to three generations in the medium. Thus, copA was suggested to be a pump uptaking copper, whereas copB is a pump for copper extrusion.

The inherited Menkes and Wilson diseases both cause a disturbance of the copper metabolism. In the X-linked Menkes disease, copper is defective to be transported into circulation system, and results in over-accumulation of copper in intestinal mucosa, kidney and connective tissue. Due to the deficiency of copper in other tissues, the activities of several copper-dependent enzymes such as lysyl and amine oxidase, tyrosinase, dopamine-b-hydroxylase and cytochrome c oxidase are affected. The clinical symptoms include progressive neuralgic degeneration, poor temperature regulation, connective tissue defects, pallor, distinctive steely or kinky hair and death in early childhood. The candidate gene for Menkes disease has been cloned (Vulpe et al., 1993). It encodes a protein with 1500 amino acids. The function of this gene is suggested to be a
pump involving copper uptake. This gene has been shown to be expressed in heart, brain, placenta, lung, muscle, kidney and pancreas, but not in the liver.

The autosomal Wilson disease results in copper toxicity. Decreased copper export from liver results in copper induced chronic liver disease and contributes to pathologic changes in other tissues, especially in brain, kidney and eye. The Wilson disease gene has been cloned almost at the same time as the Menkes gene (Tanzi et al., 1993). It encodes a protein of 1411 amino acids. In contrast to the Menkes disease gene product, the Wilson protein is speculated to be involved in copper export. This gene is most strongly expressed in liver and kidney.

The deduced amino acid sequences from copA, copB, Menkes and Wilson disease genes share a significant homology. The Wilson sequence shares 59% identity with Menkes sequence, and both share 43% and 33% identity with CopA and CopB. All four sequences exhibit the characteristic features of P-type ATPases, including a phosphorylation domain, a phosphatase domain, and the ATP binding sites. The N-terminal region contains the repeats of CXXC motif that are proposed to be Cu\(^{2+}\) binding sites. The role of the N-terminal region involved in Cu\(^{2+}\) binding is supported by the following facts: 1) CXXC motif is also present in the N-terminal region of the Cadmium-transporting ATPase of *Staphylococcus aureus*; 2) This motif resembles to the Hg\(^{2+}\) binding domain in mercuric reductase and the Cu\(^{2+}\) binding domain in metallothionein and the transcription factor ACE-1. 3) The synthetic peptide GMTCASCVHNI, which corresponds to the fourth metal-binding repeat of the Wilson's disease protein, has been shown to bind copper cation with 1:1 stoichiometry (Lutsenko, et al., 1994). Comparing
to other P-type ATPases, the newly identified copper transporting ATPases only contain six transmembrane domains, as predicted by hydrophobicity analysis. They lack additional four transmembrane domains at their C-terminus.

1.3.6 Lipid translocases

The phospholipids of animal plasma membrane are not randomly distributed across the bilayer. The aminophospholipids, phosphatidylserine and phosphatidylethanolamine, are concentrated in the inner leaflet, whereas the phosphatidylcholine and sphingomyelin are concentrated in the outer leaflet. In human red blood cells, 82% of sphingomyelin and 76% of the phosphatidylcholine is located in the outer leaflet, whereas 80% of the phosphatidylethanolamine and almost all of the phosphatidylserine is in the inner leaflet (Verkleij et al., 1973). It has been speculated that there are some enzymes referred to as lipid translocases that are responsible for the asymmetric distribution of the phospholipids (Schroit and Zwaal, 1991). A candidate enzyme, purified from erythrocytes, chromaffin granules and synaptic vesicles, was shown be a 115 kDa Mg$^{2+}$-dependent ATPase (Xie, et al., 1989; Moryama and Nelson, 1988; Morrot et al., 1990; Hicks and Parsons, 1992). Since this enzyme was copurified with the V-type ATPases from these sources, it is called ATPase II. In contrast to the V-type ATPases, this enzyme does not have the proton pumping activity; instead, it is activated by phosphatidylserine (not by phosphatidylcholine) and inhibited by vanadate. The gene encoding for bovine ATPase II has been cloned currently (Tang et al., 1996). Sequence analysis revealed that this enzyme together with the product of DSR2 gene in
yeast, ATPases of unknown function cloned from *Plasmodium falciparum* and *C. elegans* comprise a new family of P-type ATPases. A dsr2 null mutant, which was characterized to be defective in phosphatidylserine transport, further supports that this family functions as the lipid translocases.

1.4 *Tetrahymena*

*Tetrahymena thermophila* is the organism studied in this thesis. It is a free-living protozoan belonging to the phylum *Ciliophora*, subphylum *Cyrtophora*, class *Oligohymenophorea*, order *Hymenostomatida*. It is used as a simple model to study such diverse cellular activities as gene amplification and genome rearrangement, cell motility, surface antigen switch, stimulated exocytosis and intracellular patterning. Initial discoveries made in *Tetrahymena*, such as the identification of telomere structure (Blackburn, 1991), telomerase RNA and telomerase (Greider and Blackburn, 1989; Collins et al., 1995), self-splicing RNA (Cech, 1990) and the first non-muscle motor, dynein (Porter and Johnson, 1989), benefited from the unique features of this organism. The unique features of *Tetrahymena* can be summerized as follows: 1) They can grow in axenic culture at very high rates (2.5 hour cell cycle at 30°C), and to high cell densities (10^6 cells/ml) (Orias and Bruns, 1976). 2) Cells possess nuclear dimorphism. Each cell contains a germline nucleus (micronucleus) used for sexual exchange of DNA and a somatic nucleus (macronucleus) used for production of RNA to support vegetative cell growth and cell proliferation. 3) The chromosomes in macronuclei are derived from the 5 pairs of micronuclear chromosomes through the processes of chromosome amplification,
DNA fragmentation and telomere addition (Prescott, 1994). The sizes of macronuclear chromosomes are ranged from 21 kb to 1,100 kb (Altschuler and Yao, 1985; Conover and Brunk, 1986). Each chromosome has at least 40 - 50 copies. 4) *Tetrahymena* can be considered as a “free swimming neuron” because the cell responds to environmental stimuli by changing its membrane potential. Changes in membrane potential result in alterations in swimming behavior because the ciliary beating direction and frequency are both correlated with electrophysiological state of the cell. The major stimuli that *Tetrahymena* respond to are ionic, chemical, mechanical and thermal. 5) *Tetrahymena* exhibits a regulated secretory system. The mucocysts underneath the cell surface can release their storage granules in response to stimuli. In this respect, *Tetrahymena* is different from yeast that has only constitutive exocytosis and is more similar to exocrine and neuroendocrine cells in animals. 6) *Tetrahymena* possesses the osmoregulatory organelle, contractile vacuole that allows them to adapt to a variety of environmental conditions. 7) *Tetrahymena* has a well-developed oral apparatus that allows it to take up particulate food such as bacteria. The features listed in 4) through 7) are considered involving ionic regulation, however, the mechanisms remain unknown.

In my thesis, I have been focused on ion pumps in *Tetrahymena* and tried to provide some insights for above phenomena.
CHAPTER 2

P-TYPE ATPASES IN TETRAHYMENA

2.1 Introduction

P-type ATPases (ATP-driven ion-pumps) translocate various ions across the cell (and subcellular) membranes against the ion-concentration gradients. Several distinct P-type ATPases have been identified from eukaryotic organisms. The plasma membrane sodium pump (Skou, 1957) is the Na⁺- and K⁺-dependent ATPase (Na⁺/K⁺-ATPase) that undergoes a series of conformational changes during the process of Na⁺ and K⁺ translocation (Albers, 1967; Post et al., 1972). The sarcoplasmic/endoplasmic reticulum calcium-pump (Ebashi and Lipman, 1962) is the Ca²⁺-dependent ATPase (SERCA-ATPase) that also undergoes a similar reaction mechanism (Inesi and deMeis, 1989). The Na⁺/K⁺-ATPase activity is ouabain-inhibitable (Hansen, 1985), whereas the SERCA-ATPase activity is thapsigargin-sensitive (Campbell et al., 1991; Lytton et al., 1991; Sagaral et al., 1992a, b). The plasma membrane (PM) calcium pump is also a Ca²⁺-dependent ATPase, but differs from the SERCA-ATPase in its subcellular localization, insensitivity to thapsigargin, and sensitivity to calmodulin (Carafoli, 1992). The H⁺ and
Cloning and sequencing of cDNAs encoding P-type ATPases have revealed a few characteristics: (i) Within a single class of ion pumps, several isoforms with ~90 % amino acid sequence identity are found in vertebrates and plants. Three such isoforms have been identified for the SERCA-ATPase (Lytton et al., 1992), 4 isoforms for the PM Ca\(^{2+}\)-ATPase (Carafoli, 1994), 2 isoforms for the H\(^+\)/K\(^+\)-ATPase (Crowson and Shull, 1992), 3 isoforms for the Na\(^+\)/K\(^+\)-ATPase (Lingrel, 1992), and 7 isoforms for the plant PM H\(^+\)-ATPase (Ewing and Bennet, 1994). (ii) All eukaryotic P-type ATPases contain 8-10 hydrophobic regions that are assumed to form transmembrane segments, M1 - M8-10 (MacLenann et al., 1985; Schull et al., 1985; Serrano et al., 1986; Harper et al., 1989). (iii) The cytoplasmic region between M4 and M5 contains ATP-binding and phosphorylation sites. The overall sequence identity among the SERCA-, PM Ca\(^{2+}\)- and Na\(^+\)/K\(^+\)-ATPases is ~25 %, and the sequence identity between the Na\(^+\)/K\(^+\)- and H\(^+\)/K\(^+\)-ATPases is ~65 %. The conserved regions exist as clusters within the molecules, specifically in the cytoplasmic regions, which are involved in ATP-binding and
hydrolysis, allowing one to explore the phylogenetic relationships of the P-type ATPases (Green, 1992).

Search for isoforms of membrane proteins in lower eukaryotes has been beneficial to elucidate primary structure-function relationships (e.g., see a review on K⁺-channel in evolution (Salkoff and Jegla, 1995)). In this paper, we identified three distinct groups of P-type ATPases in *Tetrahymena thermophila*. One major group is similar to the Na⁺/K⁺- and H⁺/K⁺-ATPase family and consists of at least 10 isoforms. The second group includes a single member that shares a significant sequence identity with the SERCA-ATPase. The third group is composed of four distinct novel P-type ATPases which share significant homology to the *Plasmodium* ATPase-1. A phylogenetic analysis of the deduced amino acid sequences revealed the high multiplicity of *Tetrahymena* Na⁺/K⁺-ATPase (or H⁺/K⁺-ATPase) family resulting from frequent gene duplications.

2.2 Materials and Methods

2.2.1 Cells and culture condition:

*Tetrahymena thermophila* strains CU428-VII and B2086 were provided by Dr. M. A. Gorovsky (University of Rochester). Cells were maintained in 1% protease peptone medium supplemented with 10 μM FeCl₃ at room temperature and were transferred to a fresh medium every 10 days. For long term storage, cells were kept in soybean medium covered with paraffin oil to prevent evaporation. The soybean medium was made by
putting 1 soybean in 10 ml distilled water and autoclaved. Stocks can be left for up to 6 months.

For growth, cells were cultured in SPP medium (2% protease peptone, 0.2% yeast extract and 10 μM FeCl₃) in the presence of 100 units/ml penicillin and 100 μg/ml of streptomycin at 30°C unless otherwise stated. In high-salt experiment, cells growing in adaptive medium with 100 mM NaCl were transferred into 200 mM NaCl at 30°C. In some experiments, cells were incubated for 24 hour in 10 mM TE buffer (10 mM Tris-HCl, pH7.4 ; 1 mM EDTA). Although this condition would enforce nutritional and osmotic stresses on cells, this medium has been referred to as starvation medium (e.g., Guttman et al., 1980). This loosely defined term, starvation, is also used in the present study to refer to the culture in 10 mM TE buffer.

2.2.2 PCR and cloning:

A typical PCR reaction contained genomic DNA (20 ng) and primers (20 pmol each). The reaction was preheated at 94°C for 5 min and amplified for 30 cycles. Each cycle consisted of 45 seconds of denaturation at 94°C, 1 minute of annealing at 45°C and 1.5 min of extension at 72°C. The PCR product was extracted by phenol/chloroform once, precipitated by ethanol and ligated with the T-vector which was derived from pBluescript KS(-) (Marchuk et al. 1990). The positive clones were determined by blue/white selection and a subsequent PCR reamplification in which bacteria were directly placed into 10 μl of PCR reaction as template. To distinguish clones which contain the multiple genes for P-type ATPases, plasmid DNA was first double-digested
with Eco RI and Hind III in order to release inserts from pBKS(-) vector. The inserts with either internal Eco RI or Hind III or both were separated from the pool of total clones based on their distinct restriction maps. The inserts without any internal Eco RI and Hind III were separated by adding the third restriction enzyme either Eco RV or Bam HI.

2.2.3 DNA sequencing and data analysis:

The PCR products were sequenced by the dideoxy method (Sanger et al., 1977) with $^{35}$S dATP and sequenase version 2.0. Both double stranded and single stranded DNA were subject to sequencing. Double strand DNA was purified by Qiagen tips and single stranded DNA was created from pBluescript with helper phage M13K07 (Stratagene, La Jolla, CA). The sequences of TPA genes were obtained from 1-5 independent colonies in order to reduce the possibility of PCR artifacts.

We aligned the amino acid sequences using CLUSTAL V program. Gap penalty and gap length penalty were set to 12 and 14, respectively. The default PAM250 weight table was used to calculate the distance matrix.

Both the nearest neighbor-joining and maximum parsimony methods were applied to the construction of a phylogenetic tree. We used the lasergene software (DNASTAR, WI) for the nearest neighbor-joining method. The derived tree is rooted and the ancestral sequences were determined by the UPGMA algorithm. The phylogenetic tree was constructed from the alignment based on the assumption that the least distant sequences are most likely to be located on neighboring branches. We used the PAUP
software (version 3.1.1) (Swofford, 1990) for the maximum parsimony method. The shortest tree was obtained with the heuristic searching. The derived tree was unrooted. The prokaryotic sequences were defined as an outgroup. Bootstrapping was carried out for 100 times to assess the confidence of the final tree. The tree from both methods share the same topology.

2.2.4 Nucleic acid isolation and probing:

Total DNA of *T. thermophila* was isolated as follows: Cells were grown to stationary phase (1 x 10⁶ cells/ml). The cells were harvested by brief centrifugation (5,000 rpm for 5 minutes) and washed in TE (10 mM Tris, 1 mM EDTA, pH 7.5). The washed cells were resuspended in 1/20 the original volume of NET (0.5 M NaCl, 50 mM EDTA, 50 mM Tris, pH 8.5). The cells were lysed by the addition of 1% (final) Sarkosyl and were incubated at 60°C for 30 minutes. The cell lysate was extracted with the equal volume of phenol/chloroform. The nucleic acids were precipitated with an equal volume of ice cold ethanol. The precipitated nucleic acids were collected by winding out on a glass rod. The nucleic acids were resuspended in TE at about 1/1000 the original volume. RNase (DNase free) at 10 μg/ml was added and solution was incubated at 37°C for 30 minutes. The digestion is then extracted with phenol/chloroform and the DNA was precipitated with ethanol. The DNA is collected by centrifugation and washed with 70% ethanol. The final DNA was resuspended in TE with the concentration of 1 mg/ml and stored at -20°C over long periods.
For Southern blot analysis, the DNA (15 µg) was digested with appropriate restriction enzymes and fractionated by electrophoresis on 0.8% agarose. The gel was denatured in 1.5 M NaCl and 0.5 M NaOH for 1 hour and neutralized in 1.5 M NaCl and 1 M Tris (pH 8.0) for another hour. The treated gel was blotted onto a nitrocellulose membrane overnight. Before going to hybridization steps, the DNA was allowed to crosslink to nitrocellulose membranes under UV light for 15 minutes. The nitrocellulose membranes were probed with specific $^{32}$P-labeled *T. thermophila* P-type ATPase (TPA) genes at 45°C in 4 x SSPE containing formamide (50%, v/v), 0.05 M sodium phosphate buffer (pH7.0) and 5 x Denhardt's solution for overnight. The blots were sequentially washed in 4 x SSPE, in 2 x SSPE and in 1 x SSPE at 60°C and exposed to X-ray film at -70°C.

Total RNA was isolated by the acidic phenol method (Chomczynski and Sacchii, 1987). In most cases, total RNA was extracted from about $1 \times 10^7$ cells and the cell pellets were frozen in liquid nitrogen before use. The RNA isolation procedure is briefly described as follows. The *Tetrahymena* cells were lysated by addition of 0.5 ml denature solution (4 M guanidine thiocyanate, 42 mM sodium citrate, 0.83% SDS, 0.2 mM β-mercaptoethanol). The lysate was extracted with equal volume of phenol/chloroform twice. The total RNA was precipitated by adding 1/3 volume of DEPC-treated 8 M LiCl on ice for 2 hours. The RNA pellet was resuspended in 0.3 ml DEPC-treated water and precipitated with ethanol. The final RNA was resuspended in 50 µl DEPC-treated water and stored at -70°C. The concentration of RNA was determined by UV spectrophotometry (1 unit of $OD_{260} = 40$ µg/ml RNA).
Nearly 10 to 15 µg total RNA was size-fractionated on formaldehyde-agarose gel electrophoresis, blotted onto nitrocellulose membranes and probed with specific $^{32}$P-labeled TPA genes as described above.

2.2.5 RT-PCR:

1 µg of total RNA was digested with 40 U RNase-free DNase I (Boehringer) for 30 min at 37°C. After removal of the enzymes by phenol-chloroform extraction, the reverse transcription was performed as follows. The treated RNA was mixed with 5 µl 5x reaction buffer and random hexmers (3 µM), filled up with RNase-free water to 25 µl, heated to 75°C for 10 min and placed at room temperature for an additional 10 min. After the addition of 1 µl 100 mM dithiothreitol (DTT), 1 µl 25 mM deoxyribonucleoside triphosphates (dNTP) (each) and 1 µl AMV reverse transcriptase (8 U/µl; Promega, Madison, WI), samples were incubated at 42°C for 1 hour. 1 µl of the resulting cDNA was used as template for the PCR reaction performed under the above-described condition. In control, the non-reverse transcribed total RNA was used as template and no PCR products were amplified. The sequences of the oligonucleotides used are as follows:

<table>
<thead>
<tr>
<th>TPA genes</th>
<th>5' primer sequences</th>
<th>3' primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA1</td>
<td>TAATGGAGGCGATAGAA</td>
<td>CCTTTTATTTCCCTAAA</td>
</tr>
<tr>
<td>TPA2</td>
<td>TAATGGCAACCAGTCA</td>
<td>ATTTTACTCAGATAAT</td>
</tr>
<tr>
<td>TPA3</td>
<td>TAATGCTTACCTCCTA</td>
<td>CCTTTTTTACTTAGA</td>
</tr>
<tr>
<td>TPA5</td>
<td>TAATAATCAATTAAGTGT</td>
<td>CTCTCTTTTTCTCAGATT</td>
</tr>
<tr>
<td>TPA6</td>
<td>TAATAACTAATACAGCA</td>
<td>CTCTCTTTTTCTCAATC</td>
</tr>
<tr>
<td>TPA7</td>
<td>AGGCTAAAAACCTCGATAT</td>
<td>CTCTCTTTCTTCAGAG</td>
</tr>
<tr>
<td>TPA11</td>
<td>GTGTTCTTGGTCTGGGTC</td>
<td>GCTAATGCAACAGCTC</td>
</tr>
<tr>
<td>TPA12</td>
<td>CGTGAAAGGCTCTCTCTG</td>
<td>TCTGGTCTCATTCTGCG</td>
</tr>
</tbody>
</table>
The above primers are gene specific primers. The primers for TPA1-7 were selected from the two regions with the most divergent sequences. The positions of these primers are shown in Figure 2.2.

2.2.6 Pulse-field gel electrophoresis:

The intact DNA samples were prepared by embedding intact cells in low-melting agarose blocks (Altschuler and Yao, 1985; Conover and Brunk, 1986). Before embedding, the *Tetrahymena* cells were washed and suspended in 10 mM Tris (pH 7.5) for starvation. After about 20 hours of starvation, the cells were harvested and suspended at 2 x 10⁷ cells/ml in 50 mM Tris (pH 7.5) - 100 mM EDTA. An equal volume of 1.5% low-melting agarose at 37°C was added, and the mixture was cast into strips (5 x 1 mm). Upon gelling, the strips were cut into squares and incubated overnight at 50°C in buffer containing 0.5 M EDTA, 50 mM Tris (pH7.5), 0.5% Sarkosyl and 0.5 mg/ml Proteinase K (GIBCO BRL). The DNA samples were stored in 0.5 M EDTA at 4°C until needed.

The samples were run on a 1% agarose gel in 0.5 x TBE buffer which was circulated and cooled to 14°C. An auto-algorithm program in the CHEF Mapper System (Bio-Rad, Richmond, CA) was applied for DNA separation. The program was designed to separate a DNA sample with a size range of 150 - 2000 kb. The voltage gradient for the field was 6 V/cm. The switching time varied from 26.31 s to 3 m 48.48 s and the switching angle was 120°. The total running time was 29 hours. Gel blotting and hybridization was same as the routine Southern blot described above.
2.3 Results

2.3.1 Oligonucleotide design and PCR amplification:

We applied the PCR technique to clone P-type ATPase genes from *Tetrahymena thermophila*. The oligonucleotide primers were designed based on the conserved amino acid motifs of P-type ATPases: TGE site (SSLTGES), phosphorylation site (DKTGTTLT) (Shull et al., 1985) and CIR-ATP site (GDGVND) (Ovchinnikov, et al., 1987). The two pairs of primers were designed: sense TGE primer/antisense Pi primer and sense Pi primer/antisense CIR-ATP primer (Figure 2.1). The following two rules were considered for oligonucleotide designing: 1) The conserved amino acid motifs of at least 5 amino acids in a stretch were chosen. In such a case, we can design an oligonucleotide with at least 15 reliable nucleotides. The conserved amino acid stretch with an interruption by a single nonconserved amino acid is optional, but such interruption has to leave a 3 to 4 amino acid stretch at the 3' end of the oligonucleotide since a successful PCR amplification requires an oligonucleotide primer to be perfectly complemented to its target sequence, particularly at the 3' end. 2) The degenerate degree of the oligonucleotides can be reduced by considering the codon usages of organisms. In *Tetrahymena*, the synonymous codons with A or T at the third position are more frequently used than the codons with G or C at the corresponding position (Martindale, 1989). We preferred to reduce the degree of degeneracy at the 3' portion of the oligonucleotides. For sense oligonucleotides, the nucleotide at the third position of the
Figure 2.1 Diagram to illustrate the design of degenerate oligonucleotides for amplification of P-type ATPase genes from *Tetrahymena thermophila*. A 10 transmembrane model is shown on the top and the potential transmembrane segments are numbered. The conserved amino acid motifs of TGE site, phosphorylation site, FITC site and CIR-ATP binding site are indicated. The three conserved amino acid sequences used for designing the degenerate oligonucleotides are shown on the bottom and the sequences of the designed degenerate oligonucleotides are listed below.

<table>
<thead>
<tr>
<th>Oligos</th>
<th>TEG</th>
<th>Pi</th>
<th>CIR-ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>TCCTTTTAACTGGTGATC</td>
<td>TGCTCTGAAAGACTGGTAC</td>
<td>TCCAC</td>
</tr>
<tr>
<td>Antisense</td>
<td>GTIAGAGTACCAGTCTTGTC</td>
<td>TCGTTAACACCTGTCAG</td>
<td>AGA</td>
</tr>
</tbody>
</table>

last codon is usually excluded from the designed oligonucleotide. This will reduce the total degree of degeneracy by 2 to 4 fold.

We used *Tetrahymena* genomic DNA as template for PCR amplification because 1) the genomic DNA will give us opportunity to amplify all potential P-type ATPase genes from *Tetrahymena*; 2) the introns are usually small in *Tetrahymena* (Csank et al., 1990); 3) the genomic DNA is rich in genes since *Tetrahymena* macronuclei are highly polyploid and the most intervening sequences are eliminated during the development of macronucleus (Prescott, 1995). The first pair of primers (TEG/Pi) gave us a faint band
<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Number of clones</th>
<th>A/T content (%)</th>
<th>Size of macronuclear chromosomes (mb)</th>
<th>Predicted functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>TPA1</td>
<td>13</td>
<td>65.6</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TPA2</td>
<td>7</td>
<td>63.0</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TPA3</td>
<td></td>
<td>64.2</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TPA4</td>
<td>17</td>
<td>64.2</td>
<td>0.94</td>
<td>Na+/K⁺- or</td>
</tr>
<tr>
<td></td>
<td>TPA5</td>
<td>4</td>
<td>66.1</td>
<td>0.6</td>
<td>H⁺/K⁺-ATPase</td>
</tr>
<tr>
<td></td>
<td>TPA6</td>
<td>3</td>
<td>65.7</td>
<td>0.6</td>
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<tr>
<td></td>
<td>TPA7</td>
<td></td>
<td>67.0</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TPA8</td>
<td>4</td>
<td>61.7</td>
<td>0.24</td>
<td>SERCA-ATPase</td>
</tr>
<tr>
<td>II</td>
<td>TPA9</td>
<td>24</td>
<td>68.7</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TPA10</td>
<td>19</td>
<td>72.1</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>TPA11</td>
<td>1</td>
<td>69.8</td>
<td>0.4</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>TPA12</td>
<td>1</td>
<td>71.2</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1 Summary of cloning of P-type ATPase genes from *Tetrahymena thermophila*

amplification, but the second pair of primers (Pi/CIR-ATP) amplified a prominent band with a 1.1 kb size (data not shown). This DNA fragment was isolated and inserted into the unique EcoRV site in pBluescript KS(-) vector. After transformation of the host bacteria, *E. coli* XL1-blue, a total of 93 individual recombinant plasmids were randomly isolated and subjected to restriction mapping and DNA sequence analysis. Twelve distinct DNA sequences were established and were subsequently designated as TPA1-TPA12 genes (*Tetrahymena* P-type ATPases). The frequency of occurrence of the individual sequences within 93 clones varied widely; among the 93 clones, 13 were identified as TPA1, 7 as TPA2, 17 of each as TPA3 and TPA4, 4 of each as TPA5 and TPA6, 3 as TPA7, 4 as TPA8, 24 as TPA9, 19 as TPA10, and 1 of each as TPA11 and TPA12, respectively. This uneven occurrence could be due to different complimentarity
of the degenerate oligonucleotides to their target sequences. Based on the degree of
identity among the nucleotide and deduced amino acid sequences, three major groups
could be categorized (Table 2.1).

2.3.2 Identification of Distinct DNA Sequences Encoding P-type ATPases in
Tetrahymena thermophila:

The first group includes the first seven genes (TPA1 to TPA7). More than 65%
identities in their nucleotide and deduced amino acid sequences suggest that the seven
genomes in Group I belong to the same family. The second group only has a single gene,
TPA8. The third group is a mixture of the four novel genes (TPA9 to TPA12) with a
relatively low homology (below 20%).

Figure 2.2 (A - L) shows the nucleotide and deduced amino acid sequences of 12
TPA genes. All DNA sequences possess the following features of ciliate genes (Prescott,
1994): (i) DNA is A/T rich. The percentage of (A+T) content is 61.7-72.1% (Table I). (ii)
The codon usage is extremely biased. Only 44 codons are frequently used for coding
amino acids, 15 codons are rarely used, and 4 codons are not used at all. (iii) TAA and
TAG frequently appear in open reading frames (Figure 2.2). Both have been proved to
encode glutamate in ciliates rather than to be stop codons as known in other organisms
(Horowitz and Gorovsky, 1985; Hanyu et al., 1986). The deduced amino acid sequences
reveal that the cloned PCR fragments are the expected middle cytoplasmic domain of P-
type ATPases, because all of them contain the FITC site [KG(A/S)PE],
Figure 2.2 The nucleotide and deduced amino acid sequences encoding ATP-binding domains of 12 P-type ATPases (A to L) from *Tetrahymena thermophila*. The primer sequences used for PCR amplification are shown in lower case letters. The gene-specific primers used for RT-PCR are indicated by arrowlines. The underlined amino acids are conserved throughout all P-type ATPases.
Figure 2.2  (continued)
Figure 2.2  (continued)
Cont.

E. TPA5

F. TPA6

tgc tgc gac aug acc gtt gct gc1 tgc acc taa aaG tgt atc acc gtt gta agg cat atc ttc atc 60
AAT GAT ACT GCA TTA GAA ACT CTT TAT CTA TAA AAA GGT TAA GAA GCT CCT 120
N D S V F E I P N I H I D Q K G O P P
TAT GAA AAG GAA ATT GGT GTA ACG TAA AAA CCA AAT GCT GTT 180
V Y F K D I G F K I G Q A A M S S T
GCA GTT GTT GAC TGC GTT TAA GAT AAA AAT ATG GAT GTT 240
A V F D I S C O L G N V D Y I K C P
GTT ATC GAT GAT ACT GAC GAA GCT TTT AGT GGT GGA TGC ACC 300
S S S 1 Û D E E S E N S
CCT TGT AAT TAT GAA TAT GTA GAA TCA TGC TGC TAG GAC GTA AAA 360
V I N E 0 L M E R T P I L D W F T A S E
ATT ACT AAC TCT TTT TCT CTT CTT GCT CTT ATT 420
1 T N E 0 L M E R T P I L D W F T A S E

Figure 2.2 (continued)
Cont.

Figure 2.2 (continued)
Figure 2.2 (continued)
Figure 2.2

Cont.
another conserved amino acid motif for the ATP binding (Taylor and Green, 1989) (Figure 2.2).

Three putative intron sequences were found in TPA11 and TPA12 (Figure 2.2K and L). One in TPA11 is 154 bp, and the other two in TPA12 are 58 bp and 226 bp, respectively. The presence of introns is supported by the following facts: (i) Open reading frames are shifted or interrupted by the TGA stop codons in these sequences. (ii) The (A+T) content in these sequences is 78-89%, which is higher than that of the normal coding sequences in Tetrahymena (usually 60-70%) and is typical for *Tetrahymena* intron sequences (Csank et al., 1990). (iii) The consensus sequence TTAAT, which is present in the majority of *Tetrahymena* introns and is considered as the branching point for lariat formation during splicing (Csank et al., 1990), is also found in these sequences.

To judge the precise exon/intron junctions, we developed a method in which we first restrict introns between two conserved amino acid motifs by aligning their neighboring sequences with other homologous genes and then we determine 5' and 3' intron junctions in accordance with the consensus GT/AG rule. In the case of TPA11 intron, the 5' and 3' junctions are identified between two conserved motifs AGCH and GDPI (Figure 2.2K). The first intron of TPA12 is also determined by anchoring the intron between two conserved motifs KGSPE and GFRVL (Figure 2.2L). The second intron of TPA12 spans a large region between the conserved motifs MVTGD and FARMR, thus making the judgement of the exact intron junctions ambiguous. However, by determining the nucleotide sequences of the corresponding mRNA after RT-PCR and sequencing, the precise exon/intron junctions were identified.
2.3.3 Classification of *Tetrahymena* P-type ATPase Genes Based on the Amino Acid Sequence Comparisons:

The deduced amino acid sequences spanning from the phosphorylation site to the CIR-ATP site of the twelve genes (TPA1-TPA12) were compared with the corresponding sequences of known P-type ATPases.

**Group I** genes are similar to animal genes encoding the Na\(^+\)/K\(^+\) or H\(^+\)/K\(^+\)-ATPase. A striking homology between the amino acid sequences of the *Tetrahymena* Group I and animal Na\(^+\)/K\(^+\) and H\(^+\)/K\(^+\)-ATPases was found by a multiple alignment with a minimum number of gaps to maximize the matches (Figure 2.3). This alignment consists of the seven *Tetrahymena* Group I sequences and eight known Na\(^+\)/K\(^+\) and H\(^+\)/K\(^+\)-ATPase sequences from major classes of animals. The calculated overall identity was 29-37% over a stretch of 348 amino acids. The identities of the Group I sequences with other P-type ATPases (such as Ca\(^{2+}\)-ATPases) were 17-23%.

**Group II** sequence is closely related to the SERCA-ATPases. The alignment in Figure 2.4 reveals that TPA8 is closely related to SR or ER Ca\(^{2+}\)-ATPases found in animals, plants and parasitic protozoa. The identities of this gene were 29-31% with animal SERCA-ATPases, 34.7% with plant ER Ca\(^{2+}\)-ATPase, and 29.3% with the Trypanosome ER Ca\(^{2+}\)-ATPase. However the identities with other P-type ATPases including the PM Ca\(^{2+}\)-ATPase and the Na\(^+\)/K\(^+\)-ATPase were 19-21%.

**Group III** is composed of four novel genes with unknown functions. The four sequences in Group III genes (TPA9 - TPA12) share the lowest homology to any known
Figure 2.3  Alignment of amino acid sequences corresponding to the ATP-binding domains of known Na\(^+\)/K\(^+\)- and H\(^+\)/K\(^+\)-ATPases and putative *Tetrahymena* Na\(^+\)/K\(^+\)- or H\(^+\)/K\(^+\)-ATPases (TPA1 - TPA7). Shaded regions indicate that residues identical among thirteen or more ATPases. The minimum numbers of gaps (-) were introduced to maximize the alignment. RatNaK: rat Na\(^+\)/K\(^+\)-ATPase α1 subunit; ChickNaK: chicken Na\(^+\)/K\(^+\)-ATPase α1 subunit; XenoNaK: *Xenopus* Na\(^+\)/K\(^+\)-ATPase α1 subunit; TorpNaK: *Torpedo* Na\(^+\)/K\(^+\)-ATPase α subunit; DrosNaK: *Drosophila* Na\(^+\)/K\(^+\)-ATPase α subunit; CaenNaK: *Ceanorhabditis elegans* Na\(^+\)/K\(^+\)-ATPase α subunit; HydraNaK: *Hydra* Na\(^+\)/K\(^+\)-ATPase α subunit; RatHK: rat stomach H\(^+\)/K\(^+\)-ATPase α subunit. The sequence segments used for phylogenetic analysis were underlined.
Figure 2.3  (continued)
<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RatNaK</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>ChickNaK</td>
<td>IAARLNPVQSNVP----RAKCVV1SDLKMTESELDI1---------LIKHEI1--</td>
</tr>
<tr>
<td>XenoNaK</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>TorpNaK</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>DrosNaK</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>CaenNaK</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>HydraNaK</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>RatHK</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>TPA7</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>TPA6</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>TPA5</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>TPA4</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
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<tr>
<td>TPA3</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
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<tr>
<td>TPA2</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
<tr>
<td>TPA1</td>
<td>IAARLNPVQNVNP----RAKCVV1SDLKMTESELDI1---------RYKHEI1--</td>
</tr>
</tbody>
</table>

**Figure 2.3**
Figure 2.4  Alignment of amino acid sequences corresponding to the ATP-binding domains of known Ca\(^{2+}\)-ATPases and the putative *Tetrahymena* SERCA-ATPase (TPA8). LCA: tomato Ca\(^{2+}\)-ATPase; PfCa: *Plasmodium* Ca\(^{2+}\)-ATPase. Shaded regions indicate the residues of TPA8 identical to at least 3 other ATPases. Minimum numbers of gaps (−) were introduced to maximize the alignment. The sequence segments used for phylogenetic analysis were underlined.
Figure 2.5  Alignment of four blocks of amino acid sequences from the ATP-binding domains of the *Plasmodium* ATPase I, an ATPase from *C. elegans* and the four *Tetrahymena* P-type ATPase genes, TPA9 - TPA12. The conserved residues in which three out of six sequences identical are shaded. Gaps are indicated by space (-). The sequence segments used for phylogenetic analysis were underlined.
P-type ATPase sequences. The identities within this group are also low (below 20%). A blast search of GenBank database found that Group III genes share a significant homology with the *Plasmodium* ATPase-1 and an ATPase from *C. elegans*. Figure 2.5 shows an amino acid sequence comparison among the four Group III ATPases, the *Plasmodium* ATPase-1 and the *C.elegans* ATPase. Since the *Plasmodium* ATPase-1 contains several large inserted sequences within this region (Krishna et al., 1993), we eliminated some of those segments for alignment. In this alignment, most conserved residues were found flanking the following motifs: DKTGTLT (phosphorylation site), KG(S/A)PE (HTC site), (T/S)GD and GDGVND (ClR-ATP site), all of which are considered to be involved in ATP-hydrolysis reaction (Taylor and Green, 1989). The regions between these motifs showed a high rate of amino acid substitutions and insertions/deletions. The possession of the conserved amino acid motifs suggests that Group III still belongs to P-type ATPase superfamily, however the divergence in other regions implicates that Group III is a new family of P-type ATPases which may exist in all animal species.

2.3.4 Expressions of P-Type ATPases in *Tetrahymena*:

To determine whether the expression of P-type ATPase genes is regulated in a mutual-exclusive manner, the expression of the 12 distinct *Tetrahymena* P-type ATPase genes was detected by Northern blot analysis under various culture conditions [i.e., at different temperatures (25°C, 30°C, 37°C and 42°C), in high salt solution (0.2 M NaCl) and under starvation]. A single size of transcript (~3.6 kb) was detected for TPA2
Figure 2.6 Detection of mRNAs encoding Tetrahymena thermophila P-type ATPases. (A) Northern blot analysis using total RNA obtained from cells which were cultured under different conditions including at different temperatures (25°C, 30°C, 37°C, 42°C) (lanes 1 - 4), in high salt medium (0.2 M NaCl) at 30°C (lane 5) and under starvation at 30°C (lane 6). (B) RT-PCR analysis using total RNA isolated from strain CU428 cultured at 30°C. The specific mRNAs could not detected by standard Northern blot analysis were now detected by RT-PCR using gene specific oligonucleotides. In control, RT-PCR was performed without reverse transcription.
(Na\(^+\)/K\(^+\) or H\(^+\)/K\(^+\)-ATPase), TPA8 (SERCA-ATPase), and TPA9-10 (two of the Group III ATPases) under the same condition as that used in Southern blot analysis (Figure 2.6A). These four genes were constitutively expressed, and a high concentration of NaCl does not result in apparent changes in the mRNA levels (Figure 2.6A, lane 6).

However, the expression levels of these genes are highly variable under different conditions. For example, a drastic increase in the level of TPA2- and TPA10-expression can be seen at 37°C (Figure 2.6A, lane 3). Starvation down-regulates the expression of TPA2, TPA8, TPA10 genes as well as histone H4-I gene, but up-regulates the TPA9 expression (Figure 2.6A, lane 5). These results make a strong contrast to the regulation of immobilized antigen gene family in either Tetrahymena or Paramecium in which only a single gene is activated under a specific condition (e.g. at specific temperatures and in high salt medium) (Preer, 1985).

Since we detected only the mRNA for single member (TPA2) of the Group I, we further investigated the expression of the other members in this family by RT-PCR method. The two regions with the most divergent sequences within Group I family were selected to synthesize the gene-specific oligonucleotides. The total RNA was treated with DNase I before use to prevent the DNA contamination. As shown in Figure 2.6B, a major band with the expected size of 487 bp was amplified by using a pair of oligonucleotides for each gene in Group I family except TPA5.
2.3.5 Copy Number of the P-Type ATPase Genes in *Tetrahymena*:

A Southern blot analysis revealed the existence of the distinct P-type ATPase genes in *Tetrahymena* genome. The genomic DNA blots probed with isolated individual genes are shown in Figure 2.7. The hybridization was carried out under a high stringency condition (see method) in order to prevent a cross-reaction between two homologous genes. The applied condition can distinguish different genes with lower than 70% identity, and thus, identify a single copy for each of TPA1, TPA2, TPA7, TPA8, TPA10 and TPA12 (Figure 2.7A). The two-band pattern observed on the blots of TPA8 and TPA10 (Figure 2.7A, lane 1, 6 and 7) is due to the Eco RI sites within the probing regions.

However, if a gene has multiple copies in the genome which share more than 80% homology, multiple bands can be detected as seen for TPA3/TPA4, TPA5 and TPA6 (Figure 2.7 B and C). TPA3 and TPA4 are two genes sharing extremely high homology; there are only 4 amino acyl residue substitutions and 16 nucleotide substitutions within the probing region. By hybridizing with one of the two probes, TPA3, the two bands were identified on the blot when the total genomic DNA was digested with Eco RI or Hind III (Figure 2.7B, lane 1 and 3). Since both enzymes do not digest the probing region, the two bands likely represent TPA3 and TPA4, and TPA3 and TPA4 are possibly the primary products of a gene duplication event. The linkage relationship between these two genes was provided by the Bam HI digestion which only released a single ~21 kb fragment to be hybridized (Figure 2.7B, lane 9). A tentative restriction map defines that
Figure 2.7 Southern blot analysis of *Tetrahymena thermophila* genomic DNA using specific P-type ATPase gene probes encoding the ATP-catalytic domains. The total DNA from strain CU428 was digested with a set of restriction enzymes and probed with the cloned DNA fragments as indicated at the bottom of each panel. The probes used in panel (A) were TPA1, TPA2, TPA7, TPA8, TPA10 and TPA12, in panel (B) TPA3, and in panel (C) TPA5 and TPA6. In (A) and (B), fragments resulting from Eco RI (lane 1), Eco RI/Hind III (lane 2), Hind III (lane 3), Hind III/Pst I (lane 4), Pst I (lane 5), Pst I/Eco RI (lane 6), Xho I/Eco RI (lane 7), Bam HI/Eco RI (lane 8) and Bam HI (lane 9) digestion were used. Solid and open arrows indicate the fragments recognized by TPA5 and TPA6 probe, respectively. In (C), fragments resulting from Xba I (lane 1), Xba I/Eco RI (lane 2) and Eco RI (lane 3) digestion were used. The restriction map in (B) shows the linkage relationship between TPA3 and TPA4, of which the regions encoding the ATP-catalytic domains are indicated by filled box. The restriction maps in (C) represent two copies of each of TPA5 and TPA6, which are distinguishable by using a restriction enzyme, Xba I. The probing regions used in (C) are shown by filled boxes in the corresponding restriction maps, and the entire boxes including non-filled portions indicate the regions encoding the ATP-catalytic domains.
TPA3 and TPA4 are arranged in a head-to-tail direction with a 9.5 kb distance (Figure 2.7B, bottom).

The multiplicities of TPAS and TPA6 are shown in Figure 2.7C. TPAS recognizes five distinct genomic DNA fragments after an EcoRI/XbaI double digestion (no internal sites for the probing regions). The two strong TPAS-hybridizing bands (indicated by solid arrows) suggest the existence of at least two same (or similar) copies in the genome. The two weak TPAS-hybridizing bands (indicated by open arrows), which are strongly hybridized by TPA6 probe on the same blot, suggest that TPA6 also possesses at least two copies in the genome. An additional band (indicated by a solid arrow) with a high molecular weight weakly hybridizes to TPAS probe, but not to TPA6 probe, indicating the existence of another gene copy with higher homology to TPAS than to TPA6.

In conclusion, group I is a multigene family which contains at least 10 genes (TPA1-7, 2 additional genes related to TPAS and 1 additional gene related to TPA6) in the genome of *T. thermophila*, while the genes in other two groups (TPA8 and TPA9-12) do not seem to possess such multiplicity.

### 2.3.6 Chromosomal Linkage of the *Tetrahymena* P-Type ATPase Genes:

To determine whether the genes coding for P-type ATPases are linked on the same macronuclear chromosome, we used pulse-field gel electrophoresis to separate macronuclear chromosomes without enzymatic digestion (Figure 2.8A). After blotting the fractionated DNA onto nitrocellulose membranes, the blots were probed with each gene under the previously defined stringent condition (Figure 2.8B). As a result, we
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Table 2.2  Percent identities and divergencies of Na⁺/K⁺- and H⁺/K⁺-ATPases in *Tetrahymena* and other organisms
found that the *Tetrahymena* P-type ATPase genes scatter on the different chromosomes in the macronuclear genome. Although the genes in Group I family share more than 65% identities within the cloned region, they are not clustered on the same chromosome. In details, the probes for TPA1 and TPA2 hybridized to the same band with the size more than 1.9 mb respectively, suggesting that these two genes are physically linked. The probe for TPA3/4 recognized a 0.94 mb chromosome, confirming the previous conclusion that they are tandemly repeated on the same chromosome. The probes for TPA5 and TPA6 hybridized to the band with the same size, suggesting that these two genes together with their extra copies are clustered on the same chromosome (with a size of 0.6 mb). TPA7 was identified on a 0.64 mb chromosome. Thus, the members of the Group I multigene family form at least four cognate linkage groups which are distributed on at least 4 different macronuclear chromosomes. Based on the calculation using the amino acid sequences of the cloned cytoplasmic loop region (between the phosphorylation and the CIR-ATP sites) (Table 2), we found that there is a correlation between the linkage and identity of the Group I genes: the genes with more than 80% identities are linked on the same macronuclear chromosomes, while the genes with less than 70% identities are separated in different chromosomes.

The distinct chromosomal localizations of other five genes in group II and group III are also shown in Figure 2.8B, and the corresponding chromosome sizes of each gene are summarized in Table I. The inbred strains CU428 and B2086 show the identical banding pattern, indicating that the organization of macronuclear chromosomes in different strains is evolutionarily stable.
Figure 2.8  Linkage analysis of P-type ATPase genes in *Tetrahymena thermophila* macronuclei. (A) Ethidium-bromide staining of macronuclear DNA from two *T. thermophila* strains, CU428 and B2086, separated by pulse-field gel electrophoresis. (B) Linkage analysis by high stringency hybridization with the probes made from the cloned Tetrahymena P-type ATPase gene fragments. The bands on the top are unresolved micronuclear DNA. The genes of the putative Na⁺/K⁺- or H⁺/K⁺-ATPase family are located on at least four distinct macronuclear chromosomes. The genes of other TPA's are located on different macronuclear chromosomes.
2.4 Discussion

Recently, a few distinct P-type ATPase genes have been cloned from the parasitic protozoa; several putative SERCA-ATPases from *Plasmodium falciparum* (Kimura et al., 1993) and *Trypanosoma brucei* (Revalard and Pays, 1990), a putative proton pump in *Leishmania donovani* (Meade et al., 1987), and a P-type ATPase, ATPase-1, from *P. falciparum* (Krishna et al., 1993). The deduced amino acid sequences reveal high divergence from any known ATPases found in higher eukaryotes. In this study, twelve distinct P-type ATPase genes were identified in a ciliated protozoan, *T. thermophila*. A comparison of the deduced amino acid sequences with known P-type ATPases identified 3 major groups with multiple isoforms; Groups I consists of 7 isoforms and resembles Na⁺/K⁺- or H⁺/K⁺-ATPases, Group II is similar to SERCA-ATPases, and Group III contains the four novel genes which share certain homology to the Plasmodium ATPase-1 gene. All of these genes exist in macronuclei and are expressed at different levels.

2.4.1 Phylogenetic relationships of P-type ATPases:

In order to reveal the evolutionary relationship between *Tetrahymena* P-type ATPases and other known P-type ATPases, we carried out a phylogenetic analysis by using the deduced amino acid sequences of the ATP-binding domain (for detailed tree construction, see Materials and Methods). Due to the technical limitations, a heuristic scanner method instead of an exhaustive search method was used for the analysis, and the phylogenetic trees were constructed by the nearest neighbor-joining method and the
Figure 2.9 Phylogenetic tree of P-type ATPases. The tree was constructed by the neighbor-joining method based on the amino acid sequences of the ATP binding domain from 12 *Tetrahymena* P-type ATPases and from 33 known P-type ATPases that had been obtained through the GenBank and SWISS PROT(*) database using the following accession numbers: for Na⁺/K⁺-ATPase, M14511 (*Rattus norvegicus*), J03230 (*Gallus gallus*), U10108 (*Xenopus laevis*), X02810 (*Torpedo californica*), P13607* (Drosophila melanogaster), U18546 (*Caenorhabditis elegans*), M75140 (*Hydra vulgaris*); for H⁺/K⁺-ATPase, J02649 (*R. norvegicus*); for SERCA- and related ATPases, M25488 (*Saccharomyces cerevisiae* PMR1), U24069 (*S. cerevisiae* PMR2), J04703 (*Oryctolagus cuniculus*), P13585* (*G. gallus*), M62892 (*D. melanogaster*), M37087 (*Trypanosoma brucei*), M96324 (*Lycopersicon esculentum*), X71765 (*P. falciparum* ER Ca²⁺-ATPase), J03753 (*R. norvegicus* PM Ca²⁺-ATPases), L08469 (*Arabidopsis thaliana* chloroplast envelope Ca²⁺-ATPase), U20321 (*Entamoeba histolytica*), U05880 (*Paramecium tetraurelia*), L01795 (*S. cerevisiae* DSR2); for H⁺-ATPases, M24107 (*A. thaliana*), X73901 (*Dunaliella bioculata*), M17889 (*Leishmania donovoni*), J02602 (*Neurospora crassa*), M25503 (*S. cerevisiae*); for phospholipid translocases, U51100 (*Bos taurus* chromaffin granules ATPase II); U28940 (*C. elegans*), U16955 (*P. falciparum* ATPase 2), L01795 (*S. cerevisiae* DSR2); for the ATPases related to *Tetrahymena* Group III family, Z70721 (*C. elegans*), X65738 (*P. falciparum* ATPase-1), U18530 (*S. cerevisiae*); and for other ATPases, U11700 (*Homo sapiens* Cu²⁺-ATPase), U04356 (*Synechococcus sp.* Cu²⁺-ATPase), M90750 (*Bacillus firmus* Cd⁺⁺-ATPase), P18318* (*Rhizobium melliloti* FixI), K02670 (*Escherichia coli* KdpB-ATPase). The branch length values on the tree represent the distances between the sequences. The bootstrap values are shown in brackets.
maximum parsimony method. Therefore, the most parsimonious trees might have been missed. However, in both cases, the basic topology of the tree obtained (excluding *Tetrahymena* P-type ATPases) was similar to that previously reported by Green (Green, 1992).

The phylogenetic tree (Figure 2.9) depicts five major classes of P-type ATPases; the animal Na\(^+/K^+\) (or H\(^+/K^+\))-ATPases, the Ca\(^{2+}\)-ATPases, the plant and fungi H\(^+\)-ATPases, aminophospholipid translocases and prokaryotic ATPases. *Tetrahymena* Group I genes are included into the Na\(^+/K^+\) (or H\(^+/K^+\))-ATPase family. Within the Ca\(^{2+}\)-ATPase family, SERCA-ATPases and PM Ca\(^{2+}\)-ATPases cluster into their own family. The new family with undefined functions, which includes the *Plasmodium* ATPase-1, one ATPase from *C.elegans* and four *Tetrahymena* ATPases in Group III, has a closest phylogenetic relationship to aminophospholipid translocases.

The tree shown in Figure 2.9 recognizes two major steps in evolution of the *Tetrahymena* P-type ATPase genes. First, ancestral Na\(^+/K^+\) (or H\(^+/K^+\))-, Ca\(^{2+}\)- and ancestral Group III ATPase genes were separated from a primordial P-type ATPase gene, then, a large expansion of Group I - III genes occurred. The expansion of *Tetrahymena* Group I genes parallels the evolution of animal Na\(^+/K^+\) (or H\(^+/K^+\))-ATPase genes (see the discussion below).
2.4.2 The Na\(^+\)/K\(^+\) or H\(^+\)/K\(^+\)-ATPase isoform genes were newly evolved in *Tetrahymena*:

Multigene family is apparently the result of gene duplications. As shown in Figure 2.9, *Tetrahymena* Group I genes branched off the corresponding animal Na\(^+\)/K\(^+\)-ATPase lineage in an extremely early period of evolution; i.e., the branching occurred much earlier than the separation of animal Na\(^+\)/K\(^+\) and H\(^+\)/K\(^+\)-ATPase genes. As an isolated branch, this family evolved independently in *Tetrahymena*. The prominent feature of such evolution is the high frequency of gene duplications. The genes derived from the multiple duplications exhibit a similar phylogenetic pattern to the entire Na\(^+\)/K\(^+\) (or H\(^+\)/K\(^+\)) ATPase gene family as shown in Figure 2.9.

The *Tetrahymena* Group I genes have evolved through two major steps of gene duplications (Figure 2.10): duplications of linkage groups (labeled as a, b and c) which resulted in the four cognate linkage groups (i - iv), and duplications within the linkage groups (labeled as d, e and f) which resulted in TPA1 and TPA2 in Linkage group i, TPA3 and TPA4 in Linkage group ii, TPA5 and TPA6 as well as their extra copies in linkage group iii. Linkage group iv seems to be inactive in the second step duplication based on the data from both cloning and Southern blot analysis. TPA3 and TPA4 represent the more current duplication in Group I family. The current duplication for TPA3 and TPA4 is also supported by the evidence that the two genes are arranged in a head-to-tail direction with 9.5 kb distance (Figure 5B bottom). Thus, it seems to be that the evolution of Group I gene family was driven by two forces after gene duplications. One made genes to be different each other, the other made genes to separate each other.
In contrast to the rapid expansion of Group I family, Group III is stable during evolution; the four genes in Group III form a very divergent cluster. The distances among each member of Group III family are very large, indicating that each member in this family was separated at a very early stage, and then followed the four independent evolutionary lineages.

Isoforms are usually found for any proteins in higher eukaryotes. For example, α, β and γ actins and tubulins have been identified and well characterized in mammals and plants, just as in the case for Na⁺/K⁺-, Ca²⁺-and H⁺-ATPases. For each actin or tubulin (e.g., α actin), several isoforms have been found (e.g., smooth muscle-type, skeletal muscle-type and non-muscle-type α actins), just as in the case for α1, α2 and α3 isoforms of Na⁺/K⁺-ATPase. On the contrary, no isoform for actin gene (Cuppies and Pearlman, 1986) and tubulin genes (Geartig et al., 1993) have been identified in Tetrahymena. A striking difference between these structural genes and the P-type ATPase genes is that the Tetrahymena P-type ATPase gene family consists of highly complicated sub-families. For example, the Tetrahymena Group I genes include several linkage groups with at least total of 10 isoform genes, providing a case even more complicated than in vertebrate Na⁺/K⁺- (or H⁺/K⁺-) ATPase genes. In this sense, the Tetrahymena Group I genes are unique.
Figure 2.10 The phylogenetic relationship of the linkage groups in *Tetrahymena* group I family. The unrooted tree constructed by the neighbor-joining method based on the nucleotide binding domain of *Tetrahymena* P-type ATPases. The group I gene family consists of four cognate linkage groups indicated by as i-iv. The events of gene duplications between and within the linkage groups are labeled as a-f.

2.4.3 Physiological Implication:

The most obvious significance for the existence of multiple protein isoforms in multicellular organisms is that the expression of isoform genes can be regulated in a cell-type specific manner during development, and that the expressed isoform proteins can play a specific role in cell physiology after differentiation. In case of Na$^+$/K$^+$-ATPases for instance, tissue- and cell-type specific regulation of isoforms has been well documented (Herrera et al., 1988; Lingrel, 1992; Takeyasu et al., 1989). It has also been known that the pattern of isoform expression can be changed during development and under certain physiological and pathological conditions (Sweedner, 1989). Recent studies have demonstrated that some of these isoforms have distinct sensitivities to different ions (Lingrel, 1992) and transmembrane potentials (Hara et al., 1994).
One might want to extend this view of differential expression and functional diversity of vertebrate P-type ATPases to the isoforms of *Tetrahymena* P-type ATPases. It is intriguing that *Tetrahymena* can survive remarkable changes in the environment such as changes in temperature (from 17°C to 37°C) and osmolarity (5 mM to 200 mM). *Tetrahymena* may utilize related P-type ATPases to maintain intracellular ion homeostasis when challenged by widely varying environmental conditions. We have observed that the levels of mRNA for TPA2 and TPA10 are upregulated as a part of the adaptive responses to an increase in temperature. Similarly, there is a significant increase in mRNA level of TPA9 when *Tetrahymena* cells are starved. The precise functions of each of these ATPases require further detailed physiological and biochemical studies. Elucidation of the biological significance of the differential expression of ATPase isoforms within a particular homology group will subsequently depend on such studies. Since the expression of TPA9 correlates with the acidification of medium (unpublished observation), it may function as a proton pump. Recently a gene cluster of a putative Na\(^+\) pump in yeast has been analyzed (Wieland et al., 1995). The two tandemly arranged genes PMR2A and PMR2B were found to be functionally different in their tolerance to Na\(^+\) and Li\(^+\). Thus, it might be probable that even a single celled organism requires the expression of distinct ion pumps under specific conditions such as starvation, heat shock and so on.
CHAPTER 3

TPA9 AND TPA10, TWO NOVEL P-TYPE ATPases IN

TETRAHYMENA THERMOPHILA

3.1 Introduction

P-type ATPases, found in all kinds of prokaryotic and eukaryotic organisms, are critically involved in ion homeostasis in cells. Previously we demonstrated that a single-celled organism *Tetrahymena thermophila* expressed at least 12 distinct P-type ATPases which can be classified into three different families: the Na⁺/K⁺- or H⁺/K⁺-ATPase family, the SERCA-ATPase family and a novel family with undefined functions. We are interested in two members (TPA9 and TPA10) in the third family in this chapter. We report here the full-length cDNA sequences, their expression patterns as well as their subcellular localizations. Since our indirect immunofluorescence staining localized TPA9 protein and TPA10 protein to the oral apparatus and mucocysts, respectively, I will give a brief introduction to the current knowledge of these two specialized organelles in *Tetrahymena*.
In *Tetrahymena*, secretory granules are referred to as mucocysts (trichocysts in *Paramecium*) which are single membrane-bound, electron-dense, rod-shaped granules with 300 nm in diameter and 900 nm long. They contain mucin, a homogeneous mixture of acidic proteins, many of which are proteolytically processed and contain disulfide bonds (Collins and Wilhelm, 1981; Maihle and Satir, 1986). The proteins are highly concentrated, forming a paracrystalline lattice as seen by electron microscopy (Hausmann, 1978; Turkewitz et al., 1991). Mature mucocysts are found just under the plasma membrane aligning along the longitude rows between ciliary basal bodies (Tokuyasu and Scherbaum, 1965; Satir et al., 1973). When the cells are stimulated, several hundred mucocysts fuse with the plasma membrane and release their contents synchronously at the well-characterized exocytotic sites within the plasma membrane, marked by rosettes of intramembrane particles (Satir et al., 1973). The amorphous mucoid material has no defined function, though it may protect the cell from osmotic shock or from predator attacks, or it may be used for nutrition during starvation (Hausmann, 1978).

The other unique structure in *Tetrahymena* is the oral apparatus which is located at the anterior portion of the cell. It is a feeding structure composed of ciliated and non-ciliated basal bodies with the connected framework made of microtubules, microfilaments and intermediate filaments. There are about 140 basal bodies within the oral region. 110 of them are ciliated and organized into the four compound arrays: the undulating membrane bordering the right and posterior margin of the oral overtune, and three adoral zone membranelles situated on the left side (Nilsson and Williams, 1966; Forer, et al., 1970; Wolfe, 1970). The beating of the cilia around the oral region creates a
water current, together with the general swimming activity, sweeping food into the oral cavity. Food vacuoles are formed at the base of the funnel-shaped cavity. The trapped food particles including bacteria or other organic materials are ingested into the cell body with the process referred to as phagocytosis (Nilsson, 1979).

The oral apparatus can be purified with non-ionic detergents (Wolfe, 1970). Approximately 162 polypeptides were resolved from the isolated oral apparatus by two-dimensional electrophoresis (Gavin, 1980). Only a few proteins have been characterized and shown to be cytoskeleton-related (Williams, 1986; Williams et al., 1986; Honts and Williams, 1990; Keryer et al., 1990). Hauser and Hausmann (1982) found that there was an ATPase activity associated with the cytoskeleton system inside the oral basket in ciliate Pseudomicrothorax dubius by the cytochemistry method. In Tetrahymena, the isolated oral apparatus exhibits the high ATPase activity and part of the activity is due to the motor protein, myosin (Garces et al., 1995). These observations indicate that the oral apparatus functions not only as an opening channel, but also as an organelle capable of generating force for food uptake.

Our cloned P-type ATPases connecting to mucocysts and the oral apparatus are unexpected; such connection may help us to elucidate the general function of these two organelles.
3.2 Materials and Methods

3.2.1 Library screening and DNA sequencing:

A cDNA library, prepared from starved Tetrahymena RNA and cloned into the λZap vector (kindly provided by Dr. K. Karrer, Marquette University), was screened with the random-primed $^{32}$P-labeled DNA probe made from the previously identified PCR fragments of TPA9 and TPA10 (Wang and Takeyasu, 1996). The screening procedures were adopted from Stratagene's instruction manual. The filter hybridization and washing conditions were the same as those used for Southern blot hybridization.

The positive λZap phage plaques were isolated after additional two rounds screening. The single plaque was transferred to a sterile tube containing 500 μl of SM buffer and 20 μl of chloroform. The Bluescript phagemids carrying the cDNA inserts were excised by mixing 100 μl of phage stock with 200 μl of $\text{OD}_{600} = 1.0$ XL1-Blue cells and 1 μl of ExAssist helper phage. The mixture was incubated at 37°C for 15 minutes to allow absorption of phages to host cells. After addition of 3 ml of 2 x YT media, the mixture was incubated 2-2.5 hours at 37°C with shaking. The mixture was heated at 70°C for 20 minutes and the supernatant was collected after centrifugation for 15 minutes at 4,000 g. This supernatant contains the excised phagemids packaged as a filamentous phage particles, the unexcised λZap phage particles as well as helper phage particles. To rescue the excised phagemids, 1 μl of the supernatant was mixed with 200 μl of SOLR cells ($\text{OD}_{600} = 1.0$) and incubated at 37°C for 15 minutes. The mixture was then plated on
LB/Ampicillin plates (100 μg/ml) to allow the bacteria containing the Bluescript phagemids with the cDNA inserts of interest to grow.

The positive Bluescript phagemids were characterized by a double digestion with the restriction enzymes EcoRI and Xho I. These two enzymes released the whole cDNA from the pBluescript vector, thus allowing us to determine the restriction map of the cloned cDNA. The clones containing different length of cDNAs were collected and subjected to sequencing. The applied single-stranded and double-stranded DNA sequencing methods were described in Chapter 2.

3.2.2 Quantitation of mRNA:

*Tetrahymena* cells were collected and frozen in liquid nitrogen. Total RNA was isolated as described in Chapter 2. 20 μg of total RNA was loaded onto a nitrocellulose filter with the dot-blot apparatus (Bio-Rad). The filter was hybridized with the same probe used for library screening. The radioactivity of the hybridized dots was quantitated with the Phospho-Imager apparatus (Molecular Dynamics).

3.2.3 Site-directed mutagenesis and plasmid construction:

In vitro site-directed mutagenesis was performed according to the method of Kunkel (1985) with the synthetic oligonucleotides (see Appendices) which were designed to mutate *Tetrahymena* glutamate codons TAA and TAG (Horowitz and Gorovsky, 1985; Hanyu et al., 1986) into the universal glutamate codon CAA and CAG. Such conversions will allow us to express the cytoplasmic domains of cloned *Tetrahymena* P-type ATPases
in bacteria. The procedures for in vitro site-directed mutagenesis are briefly described below. The pBluescript construct was transformed into *E. coli* strain CJ 236 (*dut− ung− F′*) and the uracil-containing single-stranded DNA was isolated from this strain as the routine single-stranded DNA preparation. For each round of mutagenesis, we annealed 4-5 oligonucleotides to the uracil-containing single-stranded template simultaneously. Before the step of annealing, the synthetic oligonucleotides (100 ng each) were subjected to phosphorylation with T4 polynucleotide kinase in the presence of 1 mM ATP. The kinase reaction was incubated for 30 minutes at 37°C. The annealing reaction was incubated at 65°C for 10 minutes and then at room temperature for 20 minutes. The primer extension reaction was performed in 40 μl volume which contains 4 μl of 10 x ligation buffer, 2 μl of 2.5 mM dNTPs, 4 μl of 10 mM ATP, 1 μg of single-strand binding protein, 2 U of T4 DNA polymerase and 4 U of T4 DNA ligase (GIBCO BRL). The reaction was incubated at room temperature for 3 - 4 hours. About 10 μl of the reaction was used to transform *E. coli* DH5α and mutagenesis was confirmed by DNA sequencing.

The constructs applied for the site-directed mutagenesis contain the DNA fragments which are derived from the initial PCR cloning. The region chosen for expression of fusion proteins is located between the FITC site and the CIR-ATP site (Figure 3.6). A Bam HI site created together with the glutamate codon conversions was used to fuse this region with GST in the correct frame. The DNA fragments released by the Bam HI and Hind III or Bam HI and Kpn I double digestion were isolated by the glassmilk method and inserted into pGEX-2T or pGEX-1 vector (Pharmacia, Sweden).
3.2.4 Antibodies:

Polyclonal antibodies were raised against the GST-TPA fusion proteins. *E. coli* strain DH5α was transformed with the pGEX vectors containing the mutagenized TPA fragments. Expression and purification of the fusion proteins was performed according to the procedure described by Chapeaux et al. (1993) and Moutin et al. (1994). In details, an overnight culture of 2 ml was prepared and inoculated (in 1:20 dilution) 100 ml of the prewarmed LBA medium. Expression was induced by adding 0.1 mM IPTG at a cell density corresponding to the OD_{600} of 0.4 - 0.6. After 2 - 3 hours induction, cells were then collected, suspended in 20 ml of PBS, and vigorously sonicated at 0°C. Since the fusion proteins were insoluble, the pellet was collected and washed several times with PBS containing 2 M urea. The final pellet was examined by SDS-PAGE and about 100 ng of the partially purified fusion protein was used to immunize rabbits (Cocalico, PA). Three booster injections were carried out at one-week intervals and immunoserum was collected a week after the last boost.

<table>
<thead>
<tr>
<th>TPAs</th>
<th>Antigens*</th>
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<th>Rabbits</th>
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<th>Immunofluorescence</th>
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</tr>
<tr>
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<td>-</td>
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<td>N/A</td>
<td>N/A</td>
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</tr>
</tbody>
</table>

* The numbers of the expressed amino acid residues referring to the partial protein sequences in Figure 2.2.

Table 3.1 Summary of GST-fusion proteins and derived polyclonal antibodies

76
To purify polyclonal antibodies, GST fusion proteins were separated by 10% of SDS-PAGE and immobilized onto nitrocellulose membranes. The fusion protein bands were localized by staining with Ponceau S and marked with pencil. The membranes were blocked with 5% nonfat milk in PBS overnight. The marked protein bands were sliced out and incubated with the immunoserum in an eppendorf tube for 1 hour at room temperature. The membrane blocks were washed with a large quantity of PBS for three times. The antibodies were eluted with 100 mM glycine, pH2.5 and immediately neutralized in 1/10 volume of 1 M Tris-HCl, pH 8.5. The purified antibodies were stored at 4°C in the presence of 0.02% NaN₃.

3.2.5 Immunoblot and immunofluorescence:

For immunoblot, samples were separated in 10% of SDS-PAGE. The gels were equilibrated in the transfer buffer (25 mM Tris-HCl, 192 mM Glycine, 20% methanol) and then transferred to a nitrocellulose filter at 30 volt overnight at 4°C. The nitrocellulose filter was blocked in PBS with 5% of nonfat dry milk, incubated with immunoserum (1:1000 dilution) for 1 hour and washed with PBS plus 0.02% of Tween-20 for 3 times. The filter was incubated with the secondary antibody conjugated with alkaline phosphatase (1:2000 dilution) for another 1 hour and washed as above. Blots were developed with NBT and BCIP (GIBCO BRL).

For indirect immunofluorescence, the *Tetrahymena* cells were fixed in methanol and dropped to 12 spot slides (C.A. Hendley, Essex Ltd). Cells were permeabilized with precooled acetone at -20°C for 20 minutes. The cells were blocked with 2% BSA in PBS
for 1 hour, then incubated with the purified primary antibodies and FITC-conjugated secondary antibodies in a humid chamber for 1 hour. The slides were washed with PBS for 3 times after incubation with antibodies. Cells were examined in a Zeiss light microscope equipped with epifluorescence illumination.

3.2.6 Subcellular fractionation

*Tetrahymena thermophila* strain CU428 were grown in PPB medium at 28°C to the stationary phase with the density of 1x10^5 cells/ml and were starved in 10 mM Tris-HCl, pH 7.5 for 16 hours. Cells were harvested by centrifugation at 5,000g for 5 minutes. Prior to subcellular fractionation, cells were first subjected to deciliation by following the calcium shock procedure (Adoutte et al., 1980). The deciliated cells were fractionated according to the two phase aqueous partitioning methods (Smith and Hennessey, 1993). Briefly, cells were resuspended in cold PB buffer (250 mM sucrose, 20 mM Tris-maleate, 20 mM NaOH, pH 7.0) in the presence of protease inhibitor (1 mM PMSF, 1 mM benzimidine, 1 μg/ml leupeptine and 1 μg aprotinin). The cell suspension was dounced 40 times with a glass homogenizer and then centrifuged at 2,000g. The pellet (P1) was subjected to several washes to generate pellicle fraction (Wright and VanHouton, 1990). The supernatant (S1) was subsequently centrifuged at 15,000g for 20 min to create the crude membrane pellet which was further separated into the plasma membrane fraction (upper phase) and microsomal fraction (lower phase) at 1,000g for 5 minute by a two-phase partitioning system composed of 6% (w/w) of Dextran T500 (Pharmacia, Sweden) and 5.6% (w/w) of PEG 3350 (Sigma). The final plasma pellet and microsomal pellet
were collected by centrifuging the upper phase and lower phase at 28,000g for 20 minute after 3-4 fold dilution with PB buffer. The pellets were resuspended in a storage buffer (20 mM MOPS/Tris-HCl, pH 7.0, 25 mM KCl, 50 mM sucrose and 0.1 mM EDTA).

3.2.7 Heterologous expression of TPA9 in *Xenopus* oocytes:

Total 44 *Tetrahymena* glutamate codons TAA and TAG were converted to CAA and CAG with the synthesized oligonucleotides (Appendix A) by the site-directed mutagenesis method (Kunkel, 1985). The mutagenesis was confirmed by sequencing. The mutated insert was subcloned into the oocyte expression vector which contains a long poly A tail. The cRNA was synthesized from the linearized template with an Ambion kit (Austin, TX). Stage V-VI oocytes were obtained from a *Xenopus* female by removal of ovary segments from an anesthetized frog. Oocytes were defoliculated with 2 mg/ml collagenase (type IA, Sigma) in modified Barth’s saline (MBS) without calcium for about 1 hour. After overnight incubation in complete MBS at 18°C, oocytes were injected with 10 ng RNA (in a volume of 50 nl). The expression of injected genes was examined by Western blot after incubation for 3 days.

3.3 Results

3.3.1 The full length cDNA sequences of TPA9 and TPA10

The full length cDNAs for TPA9 and TPA10 were obtained by screening a cDNA library which was made from starved *Tetrahymena* cells. Out of about 5 x 10^4
Figure 3.1 The nucleotide and the deduced amino acid sequence of TPA9. The conserved phosphorylation site, FTTC site and CIR-ATP site in the large cytoplasmic domain as well as the ten presumable transmembrane domains were underlined.
Figure 3.2  The nucleotide and the deduced amino acid sequence of TPA10. The conserved phosphorylation site, FITC site and CIR-ATP site in the large cytoplasmic domain as well as the ten presumable transmembrane domains were underlined.
independent phage plaques, 14 and 8 positive clones for TPA 9 and TPA10 were isolated and subjected to restriction mapping. The clones with the longest inserts (~ 3.6 kb for TPA9 and ~ 3.7 kb for TPA10) were selected and sequenced. Figure 3.1 and 3.2 shows the nucleotide and the deduced amino acid sequences of TPA9 and TPA10 genes. Both cDNAs are considered to contain a full length open reading frame based on the fact that the (A+T) content in 5' and 3' untranslated regions is much higher than that in the coding regions. In the case of TPA9, the (A+T) content in the 5' and 3' untranslated region is 84% and 87%, respectively, whereas the (A+T) content in the coding region is 67.8%. In the case of TPA10, the (A+T) content in the 5' and 3' untranslated region is 95% and 87%, respectively, whereas the (A+T) content in the coding region is 71.5%. The high percentage of (A+T) content in the 5' and 3' untranslated regions was also observed in other ciliate genes (Cupple and Peariman, 1986; Karrer et al., 1993; Takemasa et al., 1989, 1990). From the above presumed open reading frames, the encoded proteins for TPA9 and TPA10 consist of 1133 amino acids and 1209 amino acids with the calculated molecular weight of 135 kDa and 139 kDa, respectively.

3.3.2 Sequence analysis:

In Chapter 2, we established that the four genes in Tetrahymena Group III family together with Plasmodium ATPase-1 and an ATPase from C. elegans constitute a new family of P-type ATPases. With the analysis of the full-length cDNA sequences of TPA9 and TPA10, we provide a few new characteristics for this new family of P-type ATPases.
Figure 3.3 Hydrophobicity plots of the four novel P-type ATPases from *P. falciparum*, *C. elegans* and *T. thermophila*. The hydrophobicity index is determined by the method of Kyte-Doolittle with a window of 20 amino acid residues. The putative transmembrane domains (shaded regions) corresponding to other P-type ATPases are numbered H1 to H10. The extra hydrophobic region residing at the N-terminal region is designated as H0.
Distance in Residues

Hydropathy Index

P. falciparum (X65738)

C. elegans (Z70721)

T. thermophila TPA10

T. thermophila TPA9
Figure 3.4 Comparison of the full length amino acid sequences from *P. falciparum* (Pf), *C. elegans* (Ce) and *T. thermophila* (9 and 10). The amino acid residues two out of four identical are shaded. The potential transmembrane domains are highlighted in yellow.
The membrane topology of this family predicted from the hydrophobicity plots (Figure 3.3) shows the common feature of the P-type ATPases in which four putative transmembrane domains at the N-terminus are separated by a long hydrophilic region from the other four or six transmembrane domains at the C-terminus. Between the first pair (H1 and H2) and the second pair (H3 and H4) of transmembrane segments, a non-hydrophobic region, proposed to be a part of the cytoplasmic phosphatase domain, is present. The unique feature of the hydrophobicity plots is the existence of an additional hydrophobic domain located at the N-terminal region. This domain (designated as H0) was found existing in TPA9, TPA10 proteins and the ATPase of *C. elegans*, but not the *Plasmodium* ATPase-1 (Figure 3.3). The amino acid sequences of this hydrophobic segment show nothing homology but hydrophobic. The charged amino acids were found flanking the this hydrophobic segment H0 (Figure 3.4). It is noteworthy that the C-terminal region of *Plasmodium* ATPase-1 and the *C. elegans* ATPases contains only four transmembrane segments, is two transmembrane segments less than TPA9 and TPA10 proteins.

The previous sequence comparison with the partial sequences (from the Pi site to CI-R-ATP site) identified several consensus motifs for this novel gene family (Figure 2.5). With the full length sequences of TPA9, TPA10, *Plasmodium* ATPase as well as *C. elegans* ATPase, we found several more conserved amino acid motifs (Figure 3.4). Among them, three consensus sequences within the phosphorylation and ATP binding domains, which are diagnostic features of P-type ATPase superfamily, is slightly
Table 3.2 Comparison of diagnostic sequences of P-type ATPases

modified from the known ion-transport ATPases (Table 3.2). In sequence 1, the amino acid just one position upstream the phosphorylated aspartate, a position invariably occupied by serine in other P-type ATPases, is a phenylalanine in four members of this family. In sequence 2, the amino acid two positions downstream the lysine residue is replaced by serine, thus the resulting FTTC sequence in this family becomes KGSPE instead of KGAPE in other P-type ATPases. In sequence 3, the valine residue at the position 4 is replaced with alanine. Another conserved amino acid motif (sequence 4) was found located within the phosphatase domain. These consensus sequences suggest that 1) the members in this family may have a common ancestor, 2) the primordial ancestral gene may have branched off the evolutionary lineage earlier than the separation of other major P-type ATPase families.

The other consensus sequences conserved within this family are dispersed throughout the whole genes, especially within the region between the transmembrane domains H1 to H8. Both N-terminal and C-terminal regions appear to be less homologous. The family specific consensus sequences were shown in Figure 3.4.
3.3.3 The expression of TPA9:

The expression of TPA9 was highly induced after *Tetrahymena* cells were transferred from the SPP growth medium into the nutrient-depleted Tris-HCl buffer (10 mM, pH 7.5). The induction level was quantitated by a dot blot analysis as shown in Figure 3.5A. The amount of TPA9 transcripts started to increase after 1 hour starvation. Nearly 10 fold increase was observed after 8 hour starvation. In control, the mRNA level of Histone H4.I gene was observed to be decreased over the same time course. Reversibly, if the starved cells were transferred back to the growth medium, the mRNA level of TPA9 was observed to return to the basal level at the same rate of induction.

To exclude the possibility that buffer itself could be an induction factor, we examined the TPA9 induction under the phosphate buffer and the double-distilled water. As shown in Figure 3.5B, all three mediums result in a significant increase in TPA9 mRNA level after 6 hour starvation. We also examined the possibility that the induction of TPA9 may be caused by the hypo-osmotic stress since the osmolarity in the starving medium is much lower than that in the growth medium. To address this issue, we starved *Tetrahymena* cells in a isotonic solution which contains 10 mM Tris-HCl plus 120 mM N-methyl-glucamine (NMG). The osmolarity of this solution was measured to be equal to that of the growth medium (120 mOsm/kg). The data in Figure 3.5B shows that the isotonic solution does not abolish the induction of TPA9 under the starvation condition, thus supports that TPA9 is a starvation-response ATPase.
Figure 3.5 Induction of TPA9. A. The time course of TPA9 induction. Cells were cultured in 10 mM Tris-HCl (pH 7.5) buffer and in the SPP medium with a supplement of 0.5% glucose. B. Effect of various starving medium on TPA9 induction. Cells were starved in double-distilled water, 10 mM Tris-HCl (pH 7.5), 10 mM phosphate buffer (pH 7.5) and 10 mM Tris-HCl plus 120 mM NMG for six hours.
The expression of TPA9 is not only induced by the depletion of nutrients, but also responded to the addition of nutrients. In Figure 3.5, a transient induction of TPA9 was observed when the late log-phase cells were supplemented with 0.5% of glucose. The expression level reached the maximum after 30 minutes of glucose addition, then rapidly dropped to the basal level within 90 minutes.

3.3.4 Immunolocalization of TPA9 and TPA10 proteins:

To obtain the polyclonal antibodies against TPA9 and TPA10 proteins, we analyzed the antigenecity of TPA9 and TPA10 based on their deduced amino acid sequences. The region between the FITC-binding site (KGSPE) and CIR-ATP binding site (GDGAND) was shown to be the region with the high antigenic potential. A 140 amino acid segment for TPA9 and a 128 amino acid segment for TPA10 from this region were selected to produce fusion protein in E. coli. The conserved amino acid motifs KGSPE and GDGAND were eliminated in order to reduce the possibility of cross-reactivity with other P-type ATPases in Tetrahymena thermophila. Since Tetrahymena uses the universal stop codons TAA and TAG to encode glutamine, all TAA and TAG codons within the selected regions of TPA9 and TPA10 were converted into the regular glutamine codons CAA and CAG. The engineered cDNA fragments were subcloned into the pGEX-2T vector (Figure 3.6). The fusion protein consists of GST at the N-terminus and the TPA9 or TPA10 segment at the C-terminus. The fusion proteins of 50 kDa (Figure 3.7A, lane 2) and 45 kDa (Figure 3.8A, lane 2) were induced in E. coli after IPTG induction. The partially purified TPA9 and TPA10 fusion proteins were used to
Figure 3.6 The plasmid construction for overexpression of GST-TPA fusion proteins. The DNA fragments encoding for the second cytoplasmic region of *Tetrahymena* P-type ATPases (shaded regions) were amplified by PCR method and subcloned into pBluescript KS(-). The oligonucleotides used to convert *Tetrahymena* glutamate codons TAA and TAG are indicated by arrows. A Bam HI site created together with the glutamate codon conversions was used to fuse the expression regions with GST in frame. The DNA fragments released by either Bam HI/Kpn I or Bam HI/Hind III double digestion were isolated by the glassmilk method and inserted into pGEX vectors.
Figure 3.7  A. Expression and purification of GST-TPA9 fusion protein. Whole cell lysate of bacteria transformed with pGEX-2T/TPA9 (from Ser^{680} to Asp^{819}) before (lane 1) and after (lane 2) IPTG induction. The expressed protein aggregated in inclusion body was solubilized with 8 M urea and the fusion protein was purified with glutathione-Sepharose 4B beads (lane 3). B. Specificity of the antiserum against TPA9 protein (arrow). Total extract from bacteria with (lane 2) or without (lane 1) IPTG induction and from unstarved (lane 3) or starved (lane 4) *Tetrahymena* cells were separated on 10% SDS-PAGE, blotted with the immunoserum raised against purified GST-TPA9 fusion protein and developed with alkaline phosphotase method.
Figure 3.8  A. The GST fusion proteins derived from TPA2 (lane 1), TPA8 (lane 3) and TPA10 (lane 2) genes were purified from inclusion bodies. B. The antisera against the purified fusion proteins recognized the native TPA2, TPA8 and TPA10 proteins in *Tetrahymena*. The plasma membrane fractions for TPA2 and TPA10, the pellicle fraction for TPA8 were separated on 10% SDS-PAGE, immunoblotted with the antisera and developed with ECL method.
immunize rabbits. Both anti-TPA9 and anti-TPA10 immunosera specifically recognize the native TPA9 and TPA10 proteins from *Tetrahymena* extract as shown in a Western blot (Figure 3.7B, lane 4 and Figure 3.8B, lane 3). The molecular weight for both TPA9 and TPA10 was measured to be about 130 kDa.

To demonstrate the localization of TPA9 and TPA10 proteins in *Tetrahymena* cells, we performed the immunofluorescence experiments. The primary antibodies for immunofluorescence were affinity-purified against the GST-TPA9 and GST-TPA10 fusion proteins (details in Materials and Methods) to reduce the background staining. Both starved and unstarved *Tetrahymena* cells were subjected to staining. In Figure 3.9 and Figure 3.10, we observed that TPA9 and TPA10 have the differential distribution pattern. TPA9 was localized to the oral apparatus at the anterior end of the cell. The four curved bands were apparently stained (Figure 3.9b). Considering the description given by Nilsson and Williams (1966), the oral apparatus was assembled by four membranelles which are composed of tightly arranged basal bodies. The longer one was referred to undulating membrane, the other three were referred to membranelles. Such a prominent fluorescence pattern for the oral apparatus was not observed in unstarved *Tetrahymena* cells. This result is consistent with our Northern blot data in which the expression of TPA9 is induced by starvation.

In contrast to TPA9, TPA10 is globally distributed on the cell surface and the fluorescent spots derived from the anti-TPA10 antibody were observed to be arranged along longitudinal rows with the punctate pattern (Figure 3.10c, d). The oral apparatus was negatively stained by anti-TPA10 antibodies. Occasionally the fixed cells were
Figure 3.9  Immunofluorescence staining of *Tetrahymena* cell with the affinity-purified TPA9 antibodies. The TPA9 protein was localized to the oral apparatus in a starved cell (b) but not an unstarved cell (a). Bar = 10 μm.
Figure 3.10  Immunolocalization of TPA10 protein in *Tetrahymena thermophila*. Phase-contrast image of a *Tetrahymena* cell (a). Immunofluorescence staining of *Tetrahymena* cells with a monoclonal antibody against α-tubulin (b) and the affinity-purified TPA10 antibodies (c and d). Both starved (c) and unstarved cells (d) were used for staining. Bar = 10 μm.
surrounded by a fluorescent halo (Figure 3.10d), resembling the capsule that has been visualized using Alcian Blue (Satir and Bleyman, 1993). The linear punctate pattern of fluorescence staining suggests that TPA10 protein is colocalized with either basal bodies or mucocysts since only these two structures have such aligning pattern on the cell cortex (Tokuyasu and Scherbaum, 1965). Based on the definition given by Allen (1967), the line where the basal bodies align is termed as the primary meridian, whereas the line between the primary meridian is designated as the secondary meridian. Mucocysts were identified aligning along the secondary meridian with the antibodies against the mucin proteins (Maihle and Satir, 1986; Turkewitz and Kelly, 1992). In order to determine the localization of TPA10 protein, we performed a double staining with a monoclonal antibody against the α tubulin. This antibody recognizes basal bodies over the cell surface, particularly the oral region was strongly stained (Figure 3.10b). Although the staining pattern of basal bodies is similar to that of TPA10 protein, the TPA10 protein is apparently not colocalized with basal bodies since 1) the staining patterns of these two antibodies do not have the same focus under the fluorescence microscopy; 2) the immunofluorescence spots of basal bodies are smaller than those stained by the TPA10 antibodies. Thus we suggest that the localization of TPA10 protein is coincident with the localization of mucocysts, a secretory organelle which is involving in secretion of mucoprotein crystals (or mucin) in Tetrahymena cells (Collins and Wilhelm, 1981; Maihle and Satir, 1986).
3.4 Discussion

Although TPA9 and TPA10 were included into the same family, the sequence identity between them is quite low, only 18%. Such low percentage of homology suggests the proteins encoded by TPA9 and TPA10 may have distinct functions. To explore the possible functions of TPA9 and TPA10, we performed the experiments on three different levels. On the first level, we investigated the environmental influence on the expression of these two genes. On the second level, we tried to identify their subcellular localizations. On the third level, we investigated their function by expressing them in Xenopus oocytes.

3.4.1 Implications from the expression and localizations of TPA9 and TPA10 proteins:

At the first level, we found that the expression of TPA9 does not respond to either temperature changes or osmolarity changes, but is regulated by the changes in nutrients. The depletion of nutrients steadily increases its mRNA level, whereas the addition of glucose causes a transient increase in its mRNA level. These data suggest that the expression of TPA9 is controlled under at least two distinct signal transduction pathways: the starvation-induced pathway and the glucose-response pathway. The former has not been well characterized even though starvation can induce large numbers of physiological changes and developmental processes. The latter is probably mediated via cAMP. The addition of glucose to the stationary-phase Tetrahymena cells was shown inducing a rapid
increase in intracellular cAMP concentration, and the resulting cAMP peak is coincident with the peak of TPA9 mRNA (Nandini-Kishore and Thompson, 1979).

Here I would like to discuss a little more on the issue of starvation, a stress that all living organisms need to deal with. Starvation is a process of energy depletion. Thus with the lack of nutrients, the problem needed to be solved by organisms is how to preserve their energy. Over the long history of evolution, a diversity of mechanisms have been adapted by organisms to protect themselves from nutrient shortage. In principle, all processes involve the cessation of cell multiplication and cell growth. The unnecessary components used for growth need to be broken down. Furthermore, some special developmental programs will be turned on, and leading cells enter a resting state in which the energy consumption is maintained at the minimum level. In yeast, starvation triggers the sporulation process (Pringle and Hartwell, 1981). In Dictyostelium, starvation initiates the aggregation process (Loomis, 1982) which transforms single-celled amoebae into a multicellular fruiting body. In Tetrahymena, the early responses of starvation include a dramatic decrease of total mRNA and proteins (Calzone et al., 1983a, b). Later on, starvation induces the sexual reproduction, i.e. conjugation process (Nanney, 1980). The significance of TPA9 protein in Tetrahymena starvation physiology is not known. This is the only P-type ATPase reported to be highly induced by starvation. All other Tetrahymena P-type ATPases are substantially down-regulated during starvation (Wang and Takeyasu, 1996). Based on the indirect immunofluorescence staining data that the induced TPA9 was highly concentrated in the opening region of the oral apparatus, we can speculate that the role of TPA9 protein could be involved in enhancing the function
of the oral apparatus. The hypothesis is that TPA9 works as a proton pump. The proton ions generated by this enzyme can be propelled into the oral cavity with the current driven by the oral cilia. The acidification inside the oral cavity may facilitate killing the trapped microorganisms, and may also facilitate the digestion of these organisms in the food vacuoles.

In terms of the secretion process, mucocysts resemble chromaffin granules from adrenal medulla, platelet dense granules, neurosecretory granules from the pituitary and cholinergic synaptic vesicles (Anderson and Orci, 1988). Like those vesicles, the pH of the interior of mucocysts has been shown to be acidic with the neutral base probe acridine orange (personal observation). The acidic pH was also observed in trichocysts in Paramecium (Garofalo and Satir, 1984). The low intragranular pH was suggested to play an important role in maintaining the mucocyst content in storage form (Garofalo and Satir, 1984). In animals, the intravesicular acidification in chromaffin granules and other granules has been known to be created by the V-type ATPase (Nelson, 1992). However, there is no evidence supporting that the acidification in mucocysts results from the same enzymes. Fok et al. (1995) found that the monospecific antibody against the 57 kDa B subunit of the V1 complex of chromaffin granules can cross-react with its counterpart subunit (66 kDa) of Paramecium V-type ATPase, but localization of this V-type ATPase was not found in trichocysts. Instead the V-type ATPase was localized to the decorated tubules of the contractile vacuole complex. Thus the mechanism for the intravesicular acidification of mucocysts or trichocysts is possibly derived from TPA10 protein.
3.4.2 The implications from the heterologous expression of TPA9:

The expression of TPA9 was not detectable in oocytes by either Western blot or ATPase assay. Codon-usage effects could be the major impediment to the efficient expression of *Tetrahymena* genes in *Xenopus* oocytes. The genes in *Tetrahymena* prefer to use the codons with either A or T at the third position, whereas the genes in *Xenopus* prefer the codons with either G or C at the third position. Particularly, the codons for leucine are extremely biased between *Tetrahymena* and *Xenopus*. As shown in Table 3.3, CTG is the most frequently used codon in the Na⁺/K⁺-ATPase gene of *Xenopus*, but it is rarely used in *Tetrahymena*. In contrast, TTA, one of frequently used codons in *Tetrahymena*, is rarely used in *Xenopus*. Codon-usage limitation was also observed in

<table>
<thead>
<tr>
<th>Codons</th>
<th>Xenopus (x 100)</th>
<th>Tetrahymena</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTG</td>
<td>60</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>CTC</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>TCG</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>CTA</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>CTA</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>TTA</td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>TCT</td>
<td>35</td>
<td>33</td>
</tr>
<tr>
<td>TCC</td>
<td>32</td>
<td>10</td>
</tr>
<tr>
<td>AGT</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>AGC</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>TCA</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>TCG</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3.3 Comparison of codon usage in *Xenopus* and *Tetrahymena*. The frequencies (x 100) of the individual leucine and serine codons were calculated from *Xenopus* Na⁺/K⁺-ATPase gene and *Tetrahymena* TPA genes.

the expression of HIV-1 envelope glycoprotein (gp120). When the gene for HIV gp120 was transfected into cultured cells, it made only a tiny amount of the protein, even if the gene was controlled by the powerful promoters. The expression of gp120 can be
improved by 8-50 fold if the biased codons of HIV gp120 were replaced with those alternatives preferred in mammalian genes (Hass et al., 1996).
CHAPTER 4

TPA2, A Na⁺/K⁺- OR H⁺/K⁺-LIKE ATPase IN

TETRAHYMENA THERMOPHILA

4.1 Introduction

The Na⁺/K⁺-ATPase uses the energy provided by ATP hydrolysis to transport Na⁺ and K⁺ ions across the plasma membrane of eukaryotic cells. The hydrolytic and transport cycles of this enzyme have been described in Chapter 1. However, because little is known about the three-dimensional structure of the enzyme, the molecular events that take place in the protein for the transport of Na⁺ and K⁺ as well as coupling of energy from the ATP hydrolysis are unknown.

Although searching for critical residues for ATP-dependent ion transport largely relies on random or site-directed mutagenesis, limitations of this approach are obvious. For example, if a given primary structure of the size of the Na⁺/K⁺-ATPase is 1,000 amino acids, there are 20,000 possible single point mutations, $4 \times 10^8$ double mutants and $1.6 \times 10^{13}$ triple mutants. The another difficulty of this approach can be summerized by the phrase of “another mutant, another problem”. The alterations of tertiary structure by
a single point mutation may cause a fairly dramatic change of function which is difficult to be explained.

To compensate for the shortcomes of the site-directed mutagenesis approach, we were searching for the divergent Na\(^+\)/K\(^+\)-ATPases existing in distantly related species. Such enzymes may provide us valuable information regarding the structure-function relationships. So far, all Na\(^+\)/K\(^+\)- and H\(^+\)/K\(^+\)-ATPases were cloned from animal species, e.g. from hydra to insects, from invertebrates to mammals. Due to evolutionary pressure, the identities among these Na\(^+\)/K\(^+\)- and H\(^+\)/K\(^+\)-ATPases are quite high. The overall identities are at least 65%. The identities within the functional domains are even higher. In this chapter, we report the full-length amino acid sequence of the most divergent Na\(^+\)/K\(^+\)- or H\(^+\)/K\(^+\)-like ATPase, TPA2, from *Tetrahymena thermophila*. The divergence in the presumable ouabain-binding sites, cation translocation sites and other functional domains are discussed.

### 4.2 Materials and Methods

The full-length TPA2 cDNA clone was obtained by screening a cDNA library with the random-primed \(^{32}\)P-labeled probe made from the previously identified PCR fragment of TPA2 (Wang and Takeyasu, 1996). The positive clones were excised in vivo following the method described in Chapter 2. The clones containing variable length of cDNAs were collected and subjected to restriction mapping and sequencing. The method for sequence analysis was also described in Chapter 2.
4.3 Results and Discussion

The full-length cDNA for TPA2 was obtained by using the previously cloned PCR fragment to screen a cDNA library made from starved *Tetrahymena* cells. Out of $2 \times 10^5$ plaques, 6 positive clones were isolated and subjected to restriction mapping (Figure 4.1).

| Figure 4.1 Restriction map of TPA2. The shaded region represents the previous cloned PCR fragment. |
|---------------------------------------------------------------|----------------------------------|
| | Eco RI | Bam HI | Xho I | Length (kb) |
| 2.0 kb | 1.6 kb |       | 3.6   |
| 2.5   | 2.3    | 2.8   | 3.3   |
| 2.0   | 2.5    | 2.8   | 3.3   |
| 1.4   | 1.4    | 1.4   | 3.6   |

All cDNA clones (except clone 5-11) share a 1.6 kb restriction fragment at their 3' ends whereas 5' portion exhibits variable length as determined by a double digestion with Bam HI and Xho I. The clone with the longest insert (about 3.6 kb) was sequenced. The size of this cDNA is close to the size of mRNA detected on the Northern blot (Figure 2.6). Figure 4.2 shows the nucleotide sequence of the cDNA and its deduced amino acid sequence. There are 87 untranslated nucleotides preceding the first potential start codon, which is in a sequence TATAAATGA, similar to the consensus sequence (A/T)AAAAATGA for initiation of translation in ciliates (Yamauchi, 1991). There are two more possible start codons in the sequences of AAGAAATGT and AGCAAATGA in frame with the first start codon which would yield the encoded protein 18 and 31 amino acids shorter. Following the stop codon TGA, there are 156 bases of 3' untranslated sequence before
Figure 4.2 The nucleotide and the deduced amino acid sequence of TPA2. The conserved phosphorylation site, FITC site and CIR-ATP site in the large cytoplasmic domain as well as the ten presumable transmembrane domains were underlined.
Figure 4.3 Hydropathy profile of TPA2 ATPase as compared with the Na⁺/K⁺- and H⁺/K⁺-ATPases from rat, Drosophila and Hydra. The hydrophobicity index is determined by the method of Kyte-Doolittle with a window of 20 amino acid residues. The putative transmembrane domains are shaded.
the poly A tail. The open reading frame beginning the first start codon has overall 64% of 
(A+T) content whereas the (A+T) contents in 5' and 3' untranslated regions are 87% and 
88%, respectively. The extraordinary (A+T) content in the 5' untranslated region 
suggests that the first ATG may be the translational initiation site. From this open 
reading frame, the encoded protein consists of 1194 amino acids with the calculated 
molecular weight of 135 kDa. The hydrophobicity plot (Figure 4.3) predicts ten potential 
transmembrane domains which are similar to those from the other P-type ATPases.

We previously aligned the partial TPA2 sequence (from the Pi site to CIR-ATP 
site) with the sequences of representative Na⁺/K⁺- and H⁺/K⁺-ATPases from major 
vertebrate and invertebrate species (Figure 2.3). Here we performed an alignment with 
the full-length amino acid sequences (Figure 4.4). The average identity between TPA2 
and the Na⁺/K⁺- and H⁺/K⁺-ATPases is nearly 36%, close to the value obtained from the 
partial sequence alignment. This value suggests that TPA2 is the most divergent Na⁺/K⁺- 
or H⁺/K⁺-ATPase identified so far. The identity is even less than that between the 
Na⁺/K⁺-ATPase and H⁺/K⁺-ATPase (the average identity between them is about 60%).
The identity between TPA2 and Na⁺/K⁺-ATPases and the identity between TPA2 and 
H⁺/K⁺-ATPases are nearly equal. TPA2 shares overall 36-37% identity to the Na⁺/K⁺- 
ATPases and 35% identity to the H⁺/K⁺-ATPases. Thus, like the result from the partial 
sequence comparison, the full-length sequence comparison still can not tell to which one 
TPA2 is closer, the Na⁺/K⁺-ATPase or the H⁺/K⁺-ATPase.

The full-length sequence alignment does reveal three distinct features for TPA2. 
First of all, the ectodomain between transmembrane segment H1 and H2 is five amino
Figure 4.4 Comparison of the full-length amino acid sequences of TPA2 with known Na⁺/K⁺- and H⁺/K⁺-ATPases. The alignment was performed with the CLUSTAL V method in which the parameters of both gap penalty and gap length penalty were set to 14. Abbreviations: Rat, Rat Na⁺/K⁺-ATPase α 1 subunit; chick, chicken Na⁺/K⁺-ATPase α 1 subunit; Xeno, Xenopus Na⁺/K⁺-ATPase α 1 subunit; Torp, Torpedo Na⁺/K⁺-ATPase α subunit; Dros, Drosophila Na⁺/K⁺-ATPase; Cean, C. elegans Na⁺/K⁺-ATPase; Hydra, Hydra Na⁺/K⁺-ATPase; HK(c), rat colon H⁺/K⁺-ATPase α subunit; HK(s), rat stomach H⁺/K⁺-ATPase α subunit. Shaded regions indicate that 8 out of 10 residues are identical to TPA2 sequence. The hydrophobic amino acid segments were underlined. The solid circles indicate the amino acid residues which were suggested to be involving in ouabain binding whereas the open circles indicate the amino acid residues which were suggested to be involving in cation translocation from site-directed mutagenesis studies.
Figure 4.4 (continued)
acid residues shorter than the corresponding region of the animal Na⁺/K⁺- and H⁺/K⁺-
ATPases. This region has been referred to as the ouabain binding domain (Palasis et al.,
1996) based on the following evidence. 1) The sequence comparison revealed that two
charged amino acid residues at the borders of this region were found only existing in
ouabain resistant Na⁺/K⁺-ATPases (e.g. Arg and Asp in rat Na⁺/K⁺-ATPase α1 subunit),
but not in ouabain- sensitive Na⁺/K⁺-ATPases from other organisms. In ouabain sensitive
Na⁺/K⁺-ATPases, the non-charged amino acids Gln/Thr and Asn appear at the same
positions. 2) Conversion of non-charged amino acids Gln and Asn of the ouabain-
sensitive sheep Na⁺/K⁺-ATPase at the N-terminal and the C-terminal borders of this
region into charged amino acids Arg and Asp conferred ouabain resistance (Price and
Lingrel, 1988). 3) The N-terminal 200 amino acids of the chicken Na⁺/K⁺-ATPase which

![Figure 4.5](image)

is shown in Figure 4.5. The transmembrane domains of *Tetrahymena* Na⁺/K⁺- or H⁺/K⁺-ATPase (TPA2).
The amino acid residues possibly involving in ouabain binding and cation translocation are
highlighted as bold letters.

113
Table 4.1 The determinant residues for ouabain binding in the Na\(^+\)/K\(^+\)-ATPase and their counterparts in TPA2.

<table>
<thead>
<tr>
<th>Residues in Na(^+)/K(^+)</th>
<th>Residues in TPA2</th>
<th>Locations</th>
<th>Site-directed mutagenesis</th>
<th>Fold resistance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-104 same H1</td>
<td>C→Y, F</td>
<td>200, 80</td>
<td>Canessa, 1992</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q-111 deleted H1-H2</td>
<td>Q, N→R, D or Q, N→D, R</td>
<td>100, 4000</td>
<td>Price, 1988</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-121 deleted H1-H2</td>
<td>D→N, E, A, S</td>
<td>50</td>
<td>Price, 1988</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-122 same H1-H2</td>
<td>Q, N→R, D or Q, N→D, R</td>
<td>100, 4000</td>
<td>Price, 1988</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-308 same H3-H4</td>
<td>Y→F, Y→C</td>
<td>20</td>
<td>Canessa, 1993</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-786 same H5</td>
<td>F→I, Q</td>
<td>11, 19</td>
<td>Palasis, 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-793 same H5-H6</td>
<td>L→P, N, K; L→I</td>
<td>24, 2.8, 8.8; 0</td>
<td>Palasis, 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-797 S H6</td>
<td>T→A, V; T→S</td>
<td>60, 70; 0</td>
<td>Feng, 1994</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-863 Y H7</td>
<td>F→L</td>
<td>5.7</td>
<td>Palasis, 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-880 K H7-H8</td>
<td>R→P</td>
<td>7.9</td>
<td>Schultheis, 1993</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The number of residues referring to the sheep Na\(^+\)/K\(^+\)-ATPase α subunit.

Table 4.2 The potential residues involved in cation translocation in the Na\(^+\)/K\(^+\)-ATPase and their counterparts in TPA2.

<table>
<thead>
<tr>
<th>Residues in Na(^+)/K(^+)</th>
<th>Residues in TPA2</th>
<th>Locations</th>
<th>Site-directed mutagenesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-327 same H4</td>
<td>E→L, Q, N</td>
<td>E→A, D</td>
<td>Kuntzweiler, 1995</td>
<td></td>
</tr>
<tr>
<td>S-775 same H5</td>
<td>S→C, A</td>
<td>S→Y</td>
<td>Arguello, 1995</td>
<td></td>
</tr>
<tr>
<td>E-779 same H5</td>
<td>E→A, Q</td>
<td>E→L, D</td>
<td>Jewell, 1993; Vilsen, 1995</td>
<td></td>
</tr>
<tr>
<td>D-804 C H6</td>
<td>D→A, L, E, N</td>
<td></td>
<td>Jewell, 1993;</td>
<td></td>
</tr>
<tr>
<td>D-808 same H6</td>
<td>D→E</td>
<td>D→L, Q, N, A</td>
<td>Jewell, 1993;</td>
<td></td>
</tr>
<tr>
<td>R-926 N H8</td>
<td>D→N, L</td>
<td></td>
<td>Jewell, 1993;</td>
<td></td>
</tr>
</tbody>
</table>

The number of residues referring to the sheep Na\(^+\)/K\(^+\)-ATPase α subunit. The (+) indicates growth in the presence of 1 mM ouabain whereas the (-) indicate no growth.
contain transmembrane domains H1, H2 and the loop region between them can confer ouabain-sensitivity on the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (Ishii and Takeyasu, 1993). Although the charged amino acids at the borders of the H1-H2 loop have been proven to be crucial for ouabain-resistance, the effect of deletions of this region on the ouabain-sensitivity has not been investigated yet. The caution to make deletion mutants is probably due to the concern of whether the mutated enzymes could be functionally active. The “natural deletion” in TPA2 indicates that the number of amino acid residues within the H1/H2 loop is not essential for the functional activity of Na\(^+/K^+\)- or H\(^+/K^+\)-ATPases.

In addition to the loop region between H1 and H2, seven amino acids in other regions were found to be equally important for ouabain binding. Those include Cys\(^{104}\) in the first transmembrane domain H1, Tyr\(^{308}\) in the H3-H4 extracellular loop, Phe\(^{786}\) in the fifth transmembrane domain H5, Thr\(^{797}\) in the sixth transmembrane domain H6, Leu\(^{793}\) in the H5-H6 extracellular loop, Phe\(^{853}\) in the seventh transmembrane domain H7 and Arg\(^{880}\) in the H7-H8 extracellular loop (see bold letters in Figure 4.5). Four of them are identical in TPA2 and the other three were substituted with the homologous amino acids in TPA2 (Table 4.1). Such substitutions were considered to have minor influence on the ouabain sensitivity. For example, when Thr\(^{797}\) was substituted with serine in the sheep Na\(^+/K^+\)-ATPase, no apparent change could be detected for ouabain sensitivity. However, if Thr\(^{797}\) was substituted with either alanine or valine, nearly 70 fold increase in ouabain resistance was observed (Feng and Lingrel, 1994). Based on these observations, we found that although five amino acids were deleted in the H1-H2 loop region, the ouabain-binding determinants in other regions remain conserved in TPA2. Thus, the evaluation of ouabain
sensitivity of TPA2 protein may help to elucidate whether the H1-H2 loop is sufficient for ouabain binding and inhibition.

Secondly, the ecodomain between transmembrane domains H7 and H8 is significantly enlarged. The region is 94 amino acid residues longer than the corresponding region of the Na+/K⁺ and H⁺/K⁺-ATPases. The 26 amino acids within this loop region have been shown to be sufficient to assemble with the glycosylated β subunit (Lemas et al., 1994). No conserved equivalent of these 26 amino acids was observed in TPA2. Three potential glycosylation sites were identified in this region (Figure 4.4).

Thirdly, the N-terminal region exhibits the most divergent sequence when comparing with the other Na+/K⁺- or H⁺/K⁺-ATPases. It is the longest N-terminal sequence among the cloned Na+/K⁺- and H⁺/K⁺-ATPases. Of 161 amino acid residues, 27 positively charged amino acids and 20 negatively charged amino acids disperse through the N-terminal region. Based on the Western blot data, the molecular weight of TPA2 is about 120 kDa which is smaller than the calculated 135 kDa. It is possible that TPA2 protein may lose some amino acids at the N-terminal region by the post-translational modification. In mammalian Na⁺/K⁺-ATPases, the first four amino acids are deleted in mature enzymes due to the post-translational modification.

In recent years, structure-function studies of Na⁺/K⁺-ATPase have focused on identifying the amino acids involved in binding and translocating Na⁺ and K⁺ ions. The negatively charged amino acids within the transmembrane domains of the Na⁺/K⁺-ATPase are the main targets for these studies. The negatively charged side chains of these amino acids are thought to neutralize the cations during transport through the hydrophobic lipid
Six oxygen-containing residues in the transmembrane domains of Na⁺/K⁺-ATPases have been implicated as essential amino acids for cations transport using site-directed mutagenesis (Jewell-Motz and Lingrel, 1993; Vilsen, 1993; 1995; Kuntzweiler et al., 1995; Arguello and Lingrel, 1995). Those include Glu^{327}, Ser^{775}, Glu^{779}, Asp^{804}, Asp^{808} and Asp^{926}. Four of them are conserved in the corresponding transmembrane domains of TPA2 as indicated by bold letters in Figure 4.5. The other two residues are replaced with the neutral amino acids in TPA2, i.e. Asp^{804} is replaced by cysteine and Asp^{926} is replaced by asparagine as shown in Table 4.2. The replacement of Asp^{804} by cysteine creates a controversy over the importance of this residue. Based on the site-directed mutagenesis data, Asp^{804} is essential for cation translocation since mutagenesis of this residue to either alanine, leucine, glutamic acid or asparagine was found to abolish the function of the Na⁺/K⁺-ATPase (Jewell and Lingrel, 1993; Vilsen, 1995). The replacement with cysteine has not been studied. Such replacement may help to elucidate the functional importance of Asp^{804}. The replacement of Asp^{926} with asparagine in TPA2 is not consistent with the mutagenesis data in which such a mutation only causes a slight change in the kinetic parameters of the Na⁺/K⁺-ATPase.
CHAPTER 5

OSMOREGULATION IN TETRAHYMENA

5.1 Introduction

Most mammalian cells have developed compensatory mechanisms to respond to the variable osmotic stress caused by changes in the concentrations of osmoactive substances or by variations in the osmolarity of the surrounding medium. Two distinct mechanisms can be recognized: the Regulatory Volume Increase (RVI), leading to a net increase of the osmolarity of the cell, and the Regulatory Volume Decrease (RVD), directing a reduction in cellular tonicity, which are activated by cell shrinkage and cell swelling, respectively. Although the RVI depends on the activation of ion pumps and carriers, the RVD response involves the concerted opening of $K^+$ and $Cl^-$ selective ionic channels, leading to a net efflux of KCl and driving the loss of cellular water.

The single-celled organism Tetrahymena has been observed having a strong ability to adapt to wide-range changes in osmolarity. Such adaptation was considered due to the special organelle, the contractile vacuole. The contractile vacuole was considered to be involved in osmoregulation by accumulating and expelling the hypotonic fluid out
of the cell (Patterson, 1980). However, the ionic composition of this fluid and how fluid segregation is achieved are not known. Recent studies show that the membranes of the contractile vacuole in protozoan such as Dictyostelium, Paramecium and Tetrahymena are enriched in V-type proton pumps (V-type ATPase) (Heuser et al., 1993; Fok et al., 1995). In Paramecium, the V-type proton ATPases are highly concentrated on the cytosolic surface of the decorated tubules which radiate from the central contractile vacuoles, as revealed by immunofluorescence microscopy (Fok et al., 1995). This has led to the hypothesis that proton ions may be the important ions in the osmoregulation of protozoan organisms. To provide evidence for the above hypothesis, we monitored the intracellular pH changes after the hypo-osmotic shock with the application of the fluorescent probe BCECF.

BCECF-AM is lipophilic acetoxyethyl (AM) ester which can freely across cell membranes. Once inside the cell, BCECF is hydrolyzed by intracellular esterases to release BCECF which is negatively charged and will be trapped. This dye is typically used as dual_excitation indicator, with the ratio of the emission intensity from excitations near the absorption maximum (505 nm) and the isosbestic point (439 nm) providing pH determination. Signal errors caused by variations in concentration, pathlength, leakage and photobleaching are canceled with this ratio method.

We observed the dramatic pH shift after hypo-osmotic shock. The data suggests that Tetrahymena may use a mechanism different from that of mammalian cells to adjust their adaptation to hypo-osmotic shock.
5.2 Materials and Methods

The stationary-staged cells were collected by centrifuging at 5,000 rpm for 1 minute. Cells were suspended in 0.5 ml of 5 mM Tris-HCl (pH 7.2) with 5 μM BCECF-AM (Molecular Probes, Eugene, OR) and incubated at room temperature for 15 minutes. Cells were washed once and resuspended in 2 ml of 5 mM Tris-HCl (pH 7.2) in a cuvette for the immediate fluorescence recording. A spectrofluorometer (Photon Technology International, CA) was applied for recording. The dual wavelength of light at 495 nm and 440 nm were used for excitation. The emission wavelength was set at 510 nm. The external calibration with the four standard pH solutions (50 mM HEPES, 90 mM NaCl with the pH 6.0, 6.4, 6.8 and 7.0) was performed prior to the experiments. The fluorescent intensities recorded at 495 nm and 440 nm were converted as pH value by Felix software.

5.3 Results and Discussion

5.3.1 The pH profile after hypo-osmotic shock

Figure 5.1 shows an observation of intracellular pH change after *Tetrahymena* cells were exposed to a hypotonic solution (i.e. 5 mM Tris-HCl, pH 7.2). The pH peak can be simply described in three phases. Phase I, a rapid pH increase; Phase II, a slow pH
Figure 5.1 The pH recording with the BCECF-loaded Tetrahymena cells. The arrows indicate the initiations of hypo-osmotic shock. The three-phase pH profile was repeated for three times. The data represents three independent experiments.

Figure 5.2 Effect of osmolarity on phase II. Phase II in the middle peak was missed when cells were pelleted and resuspended in the supernatant (red). Phase II was significantly shortened (green) when cells were resuspended in a solution containing half of supernatant and half of fresh 5 mM Tris-HCl (pH 7.2).
decrease; Phase III, a fast pH falling. The peak is reproducible after pH returning to the steady state. The following peak is slightly smaller than the previous one.

5.3.2 Na⁺/H⁺ exchange and phase II

The three-phase pH profile was induced by hypo-osmotic shock. If we centrifuge cells without changing the osmolarity of the extracellular solution, we only observed a two-phase pH profile in which phase II was missing (Figure 5.2). This indicates that phase II may be the result of hypo-osmotic shock. Based on this observation, we proposed the hypothesis that phase II results from the activation of Na⁺/H⁺ exchangers. The activation of Na⁺/H⁺ exchangers facilitates the extrusion of Na⁺ out of cells and at the same time the influx of protons decreases the intracellular pH. The extrusion of Na⁺ directly reduces intracellular osmolarity and allows cells to reach a new osmobalance. Therefore, any factors affecting the Na⁺/H⁺ exchanger activities will affect the slope of phase II.

The Na⁺/H⁺ exchanger hypothesis was supported by the following experiments: 1) the inhibition by amiloride. As shown in Figure 5.3, phase II was flatten (i.e. slope = 0) when Tetrahymena cells were preincubated with 1 mM amiloride. 2) Effect of pH on phase II. As shown in Figure 5.4, the slope of phase II was reversed when the extracellular pH was increased from 7.2 to 8.5; the slope of phase II became steeper when the extracellular pH was decreased from 7.2 to 6.0. 3) Effect of NaCl on phase II. As shown in Figure 5.5, the slope of phase II was also reversed when Tetrahymena cells were mixed with 30 mM, 60 mM and 90 mM NaCl. The data indicates that the
Figure 5.3  Inhibition effect of amiloride on the slope of phase II (the first peak). *Tetrahymena* cells were incubated with 1 mM amiloride before the hypo-osmotic shock was initiated. For the second peak, amiloride was washed away.

Figure 5.4  Effect of extracellular pH on the three-phase pH profile. The second peak was initiated (arrows) by a hypotonic solution with pH ranging from 6.0 to 8.5.
Figure 5.5 Effect of extracellular Na\(^+\) on the slope of phase II (the first peak). The slope of phase II was reverted from negative to positive when the extracellular Na\(^+\) concentration was increased. The second peak was initiated after Na\(^+\) was washed away.

Figure 5.6 The relationship of phase III and BCECF leakage. The increase of extracellular fluorescent intensity is correlated with the pH decrease in phase III. The extracellular fluorescent intensity was measured with the excitation wavelength at 439 nm and the absorption wavelength at 510 nm after cells were removed.
intracellular acidification is mainly resulted from the activation of Na⁺/H⁺ exchangers which is inhibitable by 1 mM amiloride. The elevation of extracellular pH prevents the intracellular acidification whereas the increase of extracellular Na⁺ reverses the direction of Na⁺/H⁺ exchange and results in intracellular alkalization.

5.3.3 The dye leakage and phase III

The most dramatic pH change was observed in Phase III. It is difficult to accept that such a dramatic pH decrease (sometimes ΔpH > 1 unit) could occurred in the cytoplasm. To evaluate whether phase III is the result of BCECF leakage, we monitored the fluorescent intensity in extracellular solution with the time course of pH falling in phase III. As shown in Figure 5.6, the extracellular fluorescent intensity was not increased until the phase III was initiated. The dye leakage was also supported by the evidence shown in Figure 5.4 that the ΔpH in phase III is dependent on the extracellular pH. With the increase of extracellular pH, the ΔpH is apparently decreased. Due to the significant dye leakage, the pH measured in phase III is not the intracellular pH, neither is the extracellular pH. The pH value is contributed by BCECF inside and outside cells.

5.3.4 Phase I

Phase I was created when the fresh BCECF-free buffer was used. The starting pH of phase I should reflect the real intracellular pH of Tetrahymena since there is no apparent dye leakage occurring. We proposed that the increase of pH in phase I is probably related to the V-type ATPases in the contractile vacuoles. But the evidence is
lacking. Bafilomycin, the specific inhibitor of V-type ATPases in vertebrates, did not have any effect on phase III as we observed in *Tetrahymena*. Fok et al. (1995) reported that Bafilomycin does not inhibit V-type ATPases in *Paramecium* either. Instead, they found that a new type of inhibitor, concanamycin specifically inhibits the V-type ATPase in *Paramecium* and abolishes water-outputting ability of contractile vacuoles. In this respect, the effect of concanomycin on phase I is worthwhile to be tested.
BIBLIOGRAPHY


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APPENDIX A

THE OLIGONUCLEOTIDES FOR MUTAGENESIS OF TPA9
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* The underlined sequence represents the mutagenesis point to convert TAA or TAG to CAA or CAG. All oligonucleotide sequences are complementary to coding sequence of TPA9.
APPENDIX B

THE OLIGONUCLEOTIDES FOR MUTAGENESIS OF TPA2
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a The underlined sequence represents the mutagenesis point to convert TAA or TAG to CAA or CAG. All oligonucleotide sequences represent the coding sequences of TPA2.
APPENDIX C

THE OLIGONUCLEOTIDES FOR MUTAGENESIS OF

TPA2, TPA8, TPA10, TPA11, AND TPA12
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* The underlined sequence represents the mutagenesis point to convert TAA or TAG to CAA or CAG. All oligonucleotide sequences are complementary to coding sequence of TPA8, TPA10, TPA11 and TPA12 except the oligonucleotides labeled with "*".

* The oligonucleotides were designed to create a Bam HI site.