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Genetic and Cell Biological Aspects of the Yeast Vacuolar H^+-ATPase

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The yeast vacuolar proton-translocating ATPase is a member of the third class of H^+-pumping ATPase. A family of this type of H^+-ATPase is now known to be ubiquitously distributed in eukaryotic vacuo-lysosomal organelles and archaebacteria. Nine *VMA* genes that are indispensable for expression of the enzyme activity have been cloned and characterized in the yeast *Saccharomyces cerevisiae*. This review summarizes currently available information on the *VMA* genes and cell biological functions of the *VMA* gene products.

KEY WORDS: Vacuolar H^+-ATPase; *VMA* genes; *Saccharomyces cerevisiae*.

INTRODUCTION

The fungal vacuole is an acidic compartment and plays indispensable roles in metabolic storage and in cytosolic ion and pH homeostasis, in addition to functioning in endolytic macromolecular degradation like the phagocytotic animal lysosomes (Anraku, 1987a,b; Anraku *et al.*, 1989, 1991a; Klionsky *et al.*, 1990). During the last ten years, it has become known that a new, distinct class of H^+-translocating ATPase exists ubiquitously in vacuo-lysosomal and endomembranous organelles including fungal and plant vacuoles, animal lysosomes, coated vesciles, Golgi bodies, chromaffin granules, and synaptic membrane vesicles (Al-Awqati, 1986; Anraku, 1987a,b; Bowman and Bowman, 1986; Forgac, 1989; Manolson *et al.*, 1987; Moriyama and Nelson, 1987a,b; 1989a,b,c; Nelson, 1988; Nelson and Taiz, 1989; Ohkuma, 1987; Rudnick, 1986; Stone *et al.*, 1989; Sze, 1985).

The vacuolar H^+-ATPase of the yeast *Saccharomyces cerevisiae* is a DCCD[2]-sensitive electrogenic proton pump that generates a protonmotive force of 180 mV, inside positive and acidic, in vacuolar membrane vesicles (Kakinuma *et al.*, 1981). Current studies from our laboratory have shown that the vacuolar membrane of yeast is equipped with two distinct Cl^- transport systems, which each contributes to the formation of a chemical gradient of protons across the vacuolar membrane by shunting the membrane potential generated by the H^+-ATPase (Anraku *et al.*, 1989). Vacuolar acidification is a prerequisite for operation of amino acid/H^+-antiporters (Ohsumi and Anraku, 1981; Sato *et al.*, 1984a,b), a Ca^{2+}/H^+-antiporter (Ohsumi and Anraku, 1983), and a K^+-channel (Wada *et al.*, 1987; Tanifuji *et al.*, 1988). In the case where this ability to acidify the vacuole is lost, vacuolar protein transport and nonspecific fluid phase endocytosis are considerably affected (Klionsky *et al.*, 1990; Mellman *et al.*, 1986; Umemoto *et al.*, 1990; Yamashiro *et al.*, 1990).

Vacuolar H^+-ATPases in most animal, plant, and fungal cells are large multimeric enzymes with a functional molecular mass of about 5×10^5 (E. J. Bowman *et al.*, 1986; Hirata *et al.*, 1989) and contain at least 8–9 subunits (Adachi *et al.*, 1990; Arai *et al.*, 1988; B. J. Bowman *et al.*, 1989; Kane *et al.*, 1989; Moriyama and Futai, 1990; Moriyama and Nelson, 1987a,b; Parry *et al.*, 1989; Xie and Stone, 1986). The enzymes are bafilomycin A_1-sensitive (E. J. Bowman

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[2]Abbreviations used are: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; DCCD, *N,N'*-dicyclohexylcarbodiimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; SDS, sodium dodecylsulfate.

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et al., 1988a; Umemoto *et al.*, 1990; Yoshimori *et al.*, 1991), and the reaction mechanism proposed (Hirata *et al.*, 1989; Uchida *et al.*, 1988) is similar to those for mitochondrial and bacterial F_0F_1-ATPases (Grubmeyer *et al.*, 1982; Duncan and Senior, 1985; Futai *et al.*, 1988; Noumi *et al.*, 1986).

Taiz and his coworkers first cloned and sequenced a *c*DNA encoding the carrot 69-kDa polypeptide, a catalytic subunit of the enzyme (Zimniak *et al.*, 1988). Independently and a month later, Bowman, Bowman, and their collaborators reported isolation and sequencing of two genes from *Neurospora crassa, vma1* and *vma2*, named for *v*acuolar *m*embrane *A*TPase (B. J. Bowman *et al.*, 1988; E. J. Bowman *et al.*, 1988b). These earlier contributions have provided breakthroughs for molecular biological and genetic studies of V-type H^+-ATPases.

This article addresses genetic and cell biological views of the yeast vacuolar H^+-ATPase, particularly focussing on how one can manipulate genetic screening for mutations with defective vacuolar acidification and, hence, of the *VMA* genes that affect expression of the enzyme activity in *Saccharomyces cerevisiae*.

CHARACTERIZATION OF *VMA* GENES: EARLY STUDIES

Initially, the yeast vacuolar H^+-ATPase in *S. cerevisiae* was partially purified and characterized as a three-subunit enzyme (Uchida *et al.*, 1985), like the enzymes from other organisms (B. J. Bowman *et al.*, 1985; E. J. Bowman *et al.*, 1986; Kaestner *et al.*, 1988; Lai *et al.*, 1988; Mandala and Taiz, 1985; Manolson *et al.*, 1985; Marin *et al.*, 1985; Randall and Sze, 1987; Rea *et al.*, 1988). Kane *et al.* (1989) examined the original method for purification more carefully and demonstrated that the fraction with the highest specific activity included eight polypeptides of apparent molecular masses of 100, 69, 60, 42, 36, 32, 27, and 17 kDa. They also showed that a monoclonal antibody raised against the 69-kDa polypeptide immunoprecipitated this eight-subunit enzyme, suggesting that all these polypeptides are good candidates for being subunits of the enzyme.

Based on information from the peptide and nucleotide sequences of subunits and *c*DNAs encoding the peptides of plant and mammalian counterparts, several yeast *VMA* genes have been cloned and sequenced. *VMA1* (Hirata *et al.*, 1990), *VMA2* (Anraku *et al.*, 1991a; Ohya *et al.*, 1991; Yamashiro *et al.*, 1990), *VMA3* (Nelson and Nelson, 1989; Umemoto *et al.*, 1990), and *VMA5* (Beltrán *et al.*, 1992) were cloned and characterized on the basis of partial amino acid sequences determined with the purified 67-, 57-, 16-, and 42-kDa subunits, respectively. The sequence of *VMA2* (Nelson *et al.*, 1989a,b) was determined by using a synthetic oligonucleotide derived from the counterpart *c*DNA (Manolson *et al.*, 1988). *VMA4* was accidentally discovered and characterized during a sequence study of *MIP1* (Foury, 1990).

PHENOTYPES OF *VMA*-DISRUPTED MUTANTS

Anraku and coworkers (Hirata *et al.*, 1990; Ohya *et al.*, 1991; Umemoto *et al.*, 1990) constructed chromosomal *VMA1*-, *VMA2*-, and *VMA3*-disrupted mutants and examined their growth phenotypes in detail. The three mutants can grow well in YPD medium, thereby indicating that each *VMA* gene is dispensable for growth. However, they all show a Pet^- *cls* phenotype (Table I): The mutants cannot grow on YPD plates containing 100 mM $CaCl_2$ and on YP plates containing nonfermentable carbon sources. The Pet^- phenotype was unexpected and difficult to explain at this stage of study, but the *cls* phenotype could be logically understood because the three *VMA*-disruptants have defects of vacuolar H^+-ATPase activity, ATP-dependent Ca^{2+} uptake into isolated vacuoles, and vacuolar acidification *in vivo* (Table II).

Pet^-*cls* MUTANTS AS *vma* MUTANTS

In parallel with these studies, Ohya *et al.* (1986) have isolated 30 Ca^{2+}-sensitive (*cls*) mutants of *S. cerevisiae*, each with a single recessive chromosomal mutation, and classified them into 18 complementation groups with four subtypes based on their calcium contents and Ca^{2+} uptake activities. Of these four subtypes, type IV mutants (*cls7-cls11*), which all have normal calcium contents but show increased initial rates of Ca^{2+} uptake, are *pet* mutants and this Pet^- phenotype cosegregates with the Cls^- phenotype (Ohya *et al.*, 1986).

Consequently, genetic studies were planned to determine whether *vma* mutations are allelic to some of the Pet^- *cls* mutations. The results of complementation analysis between *vma1–vma3* and *cls7–cls11*

Table I. Growth Phenotypes of Pet$^-$ *cls* and Null *vma* Mutants[a]

Medium	Wild type	*cls7* (*vma3*)	*cls8* (*vma1*)	*cls9* (*vma11*)	*cls10* (*vma12*)	*cls11* (*vma13*)	Δ*vma1*	Δ*vma2*	Δ*vma3*
YPD + 100 mM $CaCl_2$	+	−	−	−	−	−	−	−	−
YPD	+	+	+	+	+	+	+	+	+
YP-sucrose	+	+	+	+	+	+	+	+	+
YP-fructose	+	+	+	+	+	+	+	+	+
YP-lactate	+	−	−	−	−	−	−	−	−
YP-glycerol	+	−	−	−	−	−	−	−	−
YP-succinate	+	−	−	−	−	−	−	−	−

[a] Wild-type and mutant cells were streaked on the agar plates indicated. After incubation of the plates for 2 days at 30°C, growth was examined: +, a single colony was formed; −, no colony was detected. Media used were YPD (1% Bacto-yeast extract, 2% polypeptone, and 2% glucose) and YP (1% Bacto-yeast extract and 2% polypeptone with 2% of the carbon source indicated).

mutants demonstrated that *vma1* and *vma3* do not complement *cls8* and *cls7*, respectively, and that *vma2* complements all five *cls* mutants, indicating that *VMA1* and *VMA3* are identical with *CLS8* and *CLS7*, respectively. The *vma2* mutation is not involved in the *cls* mutations tested (Ohya *et al.*, 1991).

Vacuolar membrane vesicles were prepared from the five mutants, and shown to lack DCCD-sensitive ATPase activity and ATP-dependent activity for Ca^{2+} uptake (Table II). In addition, all the mutants had lost the ability to acidify their vacuoles *in vivo*. Based on these genetic and cell biological data, the Pet$^-$ *cls* mutants could be ascribable to *vma* defects. Thus *CLS9*, *CLS10*, and *CLS11* are a family of *VMA* genes and designated henceforth *VMA11*, *VMA12*, and *VMA13*, respectively (Ohya *et al.*, 1991).

Iida *et al.* (1990a,b) have demonstrated that in yeast cells the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is critically regulated at about 150 ± 80 nM in YPD medium. Measurements of $[Ca^{2+}]_i$ in individual cells of the Pet$^-$ *cls* mutants gave the mean $[Ca^{2+}]_i$ value of 900 ± 100 nM, due to the *vma* mutation (Ohya *et al.*, 1991). Thus, the 6-fold increase in $[Ca^{2+}]_i$ may trigger serious metabolic perturbation and is injurious to growth of yeast cells (Anraku *et al.*, 1991b; Galons *et al.*, 1990). The *vma* defects also result in pleiotropic effects on several cellular activities including glycerol metabolism and phospholipid metabolism (Ohya *et al.*, 1991). Unlike the majority of previously isolated *pet* mutants (Tzagoloff and Dieckmann, 1990), however, *vma* (Pet$^-$ *cls*) mutants show no detectable mitochondrial defects (Ohya *et al.*, 1991). Of interest is the observation that the *vma* mutants show inositol-dependent (Ohya *et al.*, 1991) and pH-dependent growth phenotypes (Umemoto *et al.*, 1991; Table III). These pH-dependent growth phenotypes have become known (Beltrán *et al.*, 1992; Noumi *et al.*, 1991; Yamashiro *et al.*, 1990) and will be

Table II. Vacuolar Function of Null *vma* Mutants and Pet$^-$ *cls* Mutants[a]

		ATPase activity[b]	Ca^{2+}-uptake activity[c]	Vacuolar acidification[d]
Wild-type	(YPH500)	0.67	58	+
vma1 :: URA3	(RH101)	0.03	< 0.5	−
vma2 :: TRP1	(YOV500)	< 0.01	< 0.5	−
vma3 :: URA3	(NUY29H1)	0.04	< 0.5	−
cls7	(YOC7)	< 0.01	0.9	−
cls8	(YOC102-20C)	< 0.01	0.7	−
cls9	(YOC11)	< 0.01	0.7	−
cls10	(YOC18)	< 0.01	0.8	−
cls11	(YOC28)	< 0.01	0.5	−

[a] Vacuolar membrane vesicles were isolated, and ATPase activity and Ca^{2+}-uptake were assayed (Ohya *et al.*, 1991).
[b] nmol P_i/min/mg protein.
[c] nmol Ca^{2+}/min/mg protein.
[d] Vacuolar acidification in intact cell was quantified by measuring accumulation of quinacrine in the vacuole (Umemoto *et al.*, 1990). Vacuoles were (+) or were not (−) stained with quinacrine.

Table III. Growth Phenotypes of Null *vma* Mutants[a]

Medium	ANY21 (wild-type)	YPH500 (wild-type)	NUY29H1 (Δ*vma3*)	NUY43 (Δ*vma11*)	NUY47 (Δ*vma3* Δ*vma11*)
YPD medium[b]					
pH7.0	+++	+++	−	−	−
pH5.0	+++	+++	++	++	++
pH3.0	+++	+++	++	++	++
YPD (pH5.0)					
$CaCl_2$ 100mM	+++	+++	−	−	−
$CaCl_2$ 30mM	+++	+++	−	±	−
$CaCl_2$ 10mM	+++	+++	+	+	+
$CaCl_2$ 3mM	+++	+++	++	++	++
YPD (pH5.0)					
EGTA 30mM	++	++	−	−	−
EGTA 10mM	++	+++	±	±	±
EGTA 3mM	+++	+++	++	++	++

[a]Growth phenotypes were examined as in Table I (Umemoto *et al.*, 1991).
[b]Media were buffered with 50 mM potassium phosphate or 50 mM Mes-Tris.

of potential use for selecting new genes of the *VMA* family.

If Pet$^-$ *cls* mutants are to be useful for screening the *VMA* genes, they should cover all the mutations that are directly related to vacuolar H^+-ATPase activity. Ohya *et al.* (1986, 1991) obtained five *vma* mutations from 2×10^4 mutagenized cells, in which, however, the mutations of *VMA2, VMA4*, and *VMA5* were not involved. We are currently examining the second batch of Pet$^-$ *cls* mutants from 1×10^5 mutagenized cells and have obtained a candidate *vma* mutant of a different complementation group.[3]

STRUCTURES OF THE *VMA* GENES AND PROPERTIES OF THE *VMA* GENE PRODUCTS

By 1991, nine *VMA* genes from the yeast *S. cerevisiae* have been identified (Table IV). Table IV also lists subunits encoded by the respective genes with name revised after the proposal of Nelson and Taiz (1989). Subunits represented by a capital letter are polypeptides that are peripheral in nature and are components of the vacuolar counterpart of F_1 of the ATP synthase, whereas polypeptides represented by italic letter are integral in nature and are components of the vacuolar counterpart of the F_0 sector. All the candidate subunits detected biochemically and immunochemically (Kane *et al.*, 1989) are listed as reference.

VMA1 and Vma1p (Subunit A)

VMA1. *VMA1* was isolated from a yeast genomic DNA library (Yoshihisa and Anraku, 1989) by hybridization with a 39-mer oligonucleotide probe corresponding to the amino acid sequence IVAAVSPAGGDFK in the purified 67-kDa subunit (Hirata *et al.*, 1990). The nucleotide sequence of the gene predicts a polypeptide of 1071 amino acids with a calculated molecular mass of 118,635 daltons, which is much larger than the mature form of the 67-kDa subunit in the vacuolar membrane. N- and C-terminal regions of the deduced sequence (residues 1–284 and 739–1071) are very similar to those of the catalytic subunits of vacuolar H^+-ATPases from *Daucus carota* (69 kDa) (Zimniak *et al.*, 1988) and *Neurospora crassa* (67 kDa) (E. J. Bowman *et al.*, 1988b). Alignment of the deduced sequence of yeast *VMA1* with these two sequences also revealed that it contains a nonhomologous insert of 454 amino acids (residues 285–738), which shows no detectable sequence similarities to any known ATPase subunits (Hirata *et al.*, 1990). None of the six tryptic peptides determined with the purified subunit is located in this internal region (Anraku *et al.*, 1991a; Hirata *et al.*, 1990).

The *VMA1* gene does not have any splicing consensus sequence for nuclear coded genes (Langford and Gallwitz, 1983); however, the nonhomologous region may be excised by a mechanism similar to

[3]Hayashi, M., Hirata, R., Ohya, Y., and Anraku, Y., unpublished observations.

Table IV. A Family of the *VMA* Genes That Are Indispensable for Vacuolar Acidification and Expression of the Vacuolar H^+-ATPase Activity in Yeast

Gene	Subunit	Molecular mass (kDa) Calculated[a]	Apparent[b]	Other name used	Reference
			I. Gene encoding a peripherical polypeptide		
VMA1	A	67.7	69	*TFP1*	Hirata *et al.* (1990); Shih *et al.* (1988)
VMA2	B	57.7	60	*VAT2*	Anraku *et al.* (1991a); Nelson *et al.* (1989a, b), Yamashiro *et al.* (1990)
VMA13		54.4			Ohya *et al.* (1991); Hirata *et al.*, (see footnote 7)
VMA5	C	42.3	42	*VAT5*	Beltrán *et al.* (1992)
VMA6	D		36		Bauerle *et al.* (see footnote 5)
VMA4	E	26.6	27		Foury (1990)
			II. Gene encoding an integral polypeptide		
VMA3	*c*	16.4	17		Anraku *et al.* (1991a); Nelson and Nelson (1989); Umemoto *et al.* (1990)
VMA11	*c'*	17.0		*TFP3*	Ohya *et al.* (1991); Shih *et al.* (1990); Umemoto *et al.* (1991)
VMA12		25.3			Ohya *et al.* (1991); Umemoto *et al.* (see footnote 6)
			III. Gene not identified yet		
			100		
			32		

[a]Molecular mass calculated from the deduced amino acid sequence of the respective gene.
[b]Molecular mass estimated due to SDS-polyacrylamide gel electrophoresis (see Kane *et al.*, 1989).

mitochondrial mRNA splicing (Lazowska *et al.*, 1989). Northern blotting analysis was carried out with two DNA probes; probe 1 from the homologous region of the *VMA1* gene and probe 2 from the nonhomologous insert. Each probe detected only a single RNA species of 3.5 kb in both poly(A)$^+$ and total RNA fractions (Hirata *et al.*, 1990), which is consistent with the whole length of the *VMA1* open reading frame (3213 bases). This 3.5-kb species was not observed in the RNA fraction from the null *vma1* cells. Thus, it was concluded that the transcript of *VMA1* is not spliced and that a novel processing mechanism, which may involve a post-translational excision of the integral region followed by peptide ligation, operates on the yeast *VMA1* product (Hirata *et al.*, 1990). Recently, Kane *et al.* (1990) have shown that yeast cells carrying *VMA1* under control of the inducible *GAL10* promoter express a 119-kDa polypeptide of the unprocessed *VMA1* gene product in galactose medium and that the precursor undergoes a post-translational cleavage and splicing to yield the mature 67-kDa subunit A and the 50-kDa polypeptide.

The 5′-flanking region of the *VMA1* open reading frame contains a TATA-like sequence (TATAGATA at position −79) and the consensus sequences for the yeast transcription initiation site (RRYRR at positions −24 and −20; R and Y represent purine and pyrimidine, respectively; Hahn *et al.*, 1985). In the 3′-flanking region, a set of sequences homologous to the predicted transcription termination signal of yeast (Zaret and Sherman, 1982) is found (Hirata *et al.*, 1990).

Assuming that the whole stretch of the nonhomologous insert (residues 285–738) is removed from the *VMA1* product, a molecular mass of the mature subunit consisting of 617 amino acids is calculated to be 67,722 daltons. This is in good agreement with the value of 67 kDa estimated by SDS-polyacrylamide gel electrophoresis (Hirata *et al.*, 1990). The deduced primary sequence of yeast Vma1p is very similar to those of the *Neurospora crassa* (E. J. Bowman *et al.*, 1988b) and *Daucus carota* (Zimniak *et al.*, 1988) counterparts: About 73% and 60% of the residues are identical with the fungal and plant sequences, respectively.

Shih *et al.* (1988) isolated a dominant trifluoperazine-resistant mutant, *TFP1-408*, and determined the nucleotide sequence of the mutated gene. Comparison of the nucleotide sequences of the *VMA1* and *TFP1-408* genes indicates that the two genes are identical (Hirata *et al.*, 1990). Both genes are located on chromosome IV and the dominant phenotype of *TFP1-408* can be explained by the oligomeric nature of subunit A in the H^+-ATPase complex.

Vma1p (Subunit A). Uchida *et al.* (1988) have shown that Vma1p is the catalytic subunit of the enzyme complex by experiments with affinity labeling

and chemical modification of the purified enzyme: (1) The ATP analogue 8-azido ATP binds to subunit A specifically in an ATP-inhibitable manner. (2) NBD-Cl, which is known to interact specifically with the β subunit (a catalytic polypeptide) of the mitochondrial ATP synthase, can bind covalently to the subunit in an ATP-protectable manner. (3) This chemical reaction, which modifies a single tyrosine residue in the molecule, results in enzyme inactivation and loss of the ability to form an enzyme–ATP complex, the first intermediate in the hydrolytic reaction (Hirata *et al.*, 1989).

Consistent with these biochemical data, the deduced primary sequence of Vma1p shows about 25% sequence identity over 400 residues with β subunits of F_0F_1-ATPases (Hirata *et al.*, 1990). Vma1p has consensus sequences for nucleotide-binding domains proposed by Walker *et al.* (1982) and, in addition, contains conserved amino acid residues that have proven to be important for ATP hydrolysis (Futai *et al.*, 1989): Gly-142, Gly-149, Gly-154, Lys-155, Asp-242, and Arg-246 in the *E. coli* β subunit are all conserved, suggesting that the catalytic subunits from the two classes of ATPase share a similar structure and mechanism of ATP hydrolysis (Duncan and Senior, 1985; Grubmeyer *et al.*, 1982; Hirata *et al.*, 1989; Noumi *et al.*, 1986; Uchida *et al.*, 1988).

VMA2 and Vma2p (Subunit B)

VMA2. Based on the nucleotide sequence information of the 57-kDa subunit of *Arabidopsis thaliana* vacuolar H^+-ATPase (Manolson *et al.*, 1988), Nelson *et al.* (1989a) isolated a *c*DNA clone encoding a counterpart subunit in yeast. The original nucleotide sequence was revised (Nelson *et al.*, 1989b; Yamashiro *et al.*, 1990), and the predicted amino acid sequence deduced from the revised nucleotide sequence has proven to contain all the four peptides that were determined with the purified 57-kDa subunit from *S. cerevisiae* (Anraku *et al.*, 1991a; Hirata *et al.*, 1990). Independent of these studies, Ohya *et al.* (1991) isolated the *VMA2* gene and showed by Western blotting analysis that the null *vma2* strain has no immunoreactive 57-kDa subunit in the cell lysate.

The nucleotide sequence of *VMA2* predicts a polypeptide of 517 amino acids with a calculated molecular mass of 57,749 daltons (Yamashiro *et al.*, 1990). Comparison of sequence homology (Yamashiro *et al.*, 1990) revealed extensive sequence identities of 82, 74, 54, 58, and 74%, respectively, to the 60-kDa subunits from *Neurospora crassa* (B. J. Bowman *et al.*, 1988), *Arabidopsis thaliana* (Manolson *et al.*, 1988), *Sulfolobus acidocaldarius* (Denda *et al.*, 1988), *Methanosarcina barkeri* (Inatomi *et al.*, 1989), and human endomembrane (Südhof *et al.*, 1989).

Vma2p (Subunit B). Vma2P seems to be present in an equimolar amount with Vma1p in the purified enzyme from yeast (Uchida *et al.*, 1985; Kane *et al.*, 1989). Subunit B does not bind 8-azido ATP and NBD-Cl under conditions in which they bind to subunit A (Uchida *et al.*, 1988). Vacuoles isolated from the *vma2* cells showed no vacuolar H^+-ATPase activity and no vacuolar acidification (Ohya *et al.*, 1991; Yamashiro *et al.*, 1990), so this subunit is essential for the expression of enzyme activity, probably functioning as a regulatory component (Hirata *et al.*, 1989).

VMA3 and Vma3p (Subunit *c*)

VMA3. Two independent strategies were taken for isolation and cloning of the *VMA3* gene. For a hybridization probe to *VMA3*, Nelson and Nelson (1989) synthesized a 105-mer oligonucleotide according to 35 amino acids of the C-terminus of the 17-kDa proteolipid from bovine chromaffin granules (Mandel *et al.*, 1988) and isolated two positive clones by dot blots and Southern hybridization. Umemoto *et al.* (1990) isolated and characterized one positive clone, using a 43-mer oligonucleotide probe that was synthesized based on the determination of the N-terminal 17 amino acids with the purified 16-kDa proteolipid from yeast vacuoles. In both cases, the codons most frequently preferred in yeast (Maruyama *et al.*, 1986) were chosen. Nucleotide sequencing of all the candidates revealed that they contain a single open reading frame encoding a hydrophobic polypeptide of 160 amino acids with a calculated molecular mass of 16,350 (Nelson and Nelson, 1989; Umemoto *et al.*, 1990). Umemoto *et al.* (1990) have found that the 5′-upstream region of the transcription site in the *VMA3* gene contains a TATA-like structure, TATAAAAA, at position -95 and the consensus sequences for the yeast transcription initiation site (RRYRR at positions -26 and -23; Hahn *et al.*, 1985). In the 3′-untranslated region, a set of sequence homologous to the consensus termination signal (Zaret and Sherman, 1982) starts at position 512. *VMA3* (*CLS7*) has been mapped on the left arm of chromosome V in *S. cerevisiae* (Ohya *et al.*, 1986).

The predicted amino acid sequence of the *VMA3* gene product shows extensive sequence identity (64%)

to the 17-kDa proteolipid from bovine chromaffin granules (Mandel *et al.*, 1988), but is less homologous (30% identity) to the proteolipid from *Sulfolobus acidocaldarius* (Denda *et al.*, 1989). The amino acid sequence of the N-terminal half of Vma3p (residues 1–78) was found to be 23% identical to that of the C-terminal half (residues 79–160) (Umemoto *et al.*, 1990): Homology search in the *VMA3* DNA sequence revealed that the former fragment (base positions 1–224) is 54% identical to the latter (base positions 225–480). The C-terminal half of yeast Vma3p shows significant homology (about 35% identity) to 8-kDa proteolipids of spinach chloroplasts, yeast mitochondria, bovine mitochondria, and cyanobacterium *Synechococcus* (Cozens and Walker, 1987; Sebald and Hoppe, 1981). Homology of the N-terminal half is less marked and shows about 27% identity to 8-kDa proteolipids of thermophilic bacterium PS3 and *Bacillus megaterium* (Brusilow *et al.*, 1989; Sebald and Hoppe, 1981). These facts suggest that the yeast *VMA3* gene is a duplicated and diverged form of the genes encoding the 8-kDa proteolipid of the F_0-sector of the F_0F_1-ATPase (Nelson and Nelson, 1989).

Vma3p (Subunit *c*). Subunit *c* has been identified as a DCCD-binding protein (Uchida *et al.*, 1985) and has been suggested to function as a channel for proton translocation in the H^+-ATPase complex (Kakinuma *et al.*, 1981). Hydropathy analysis (Kyte and Doolittle, 1982) predicts that Vma3p contains four membrane-spanning domains (Nelson and Nelson, 1989; Umemoto *et al.*, 1990): Glu-137 exists in the fourth domain, which has been reported to be the conserved DCCD-binding site in various proteolipids of the F_0F_1-ATPases.

As mentioned above, NBD-Cl is shown to inhibit the formation of an enzyme–ATP complex. By contrast, DCCD does not affect the binding of ATP to a high-affinity catalytic site (Uchida *et al.*, 1988). In other words, DCCD does not inhibit the activity of the single-cycle hydrolysis of ATP but inhibits strongly that of the multi-cycle hydrolysis of ATP as well as H^+-translocation across the vacuolar membrane (Hirata *et al.*, 1989).

VMA4 and Vma4p (Subunit E)

VMA4. Foury (1990) uncovered *VMA4* while characterizing the *MIP1* gene that encodes the catalytic subunit of the yeast mitochondrial DNA polymerase (Foury, 1989). The *VMA4* open reading frame (699 bases) predicts a hydrophilic polypeptide of molecular mass 26.6 kDa. The deduced amino acid sequence shows 34% identity to the 31-kDa subunit of the vacuolar H^+-ATPase from kidney microsomes (Hirsch *et al.*, 1988). In the 5′-flanking region of *VMA4*, a perfect TATATA box is located 93 bp upstream of the UAG codon, and about 250 bp upstream of the AUG initiation codon, two sequences which exhibit hight homology with the RPG and Homoll UAS elements (Leer *et al.*, 1985; Teem *et al.*, 1984) are present. To these sequences the TUF(*RAP1/GRF1*) transcriptional factor (Vignais *et al.*, 1987) binds specifically and is involved in the activation of the transcription of the *VMA4* gene. *VMA4* and *MIP1* are located on chromosome XV and the initiation sites of their mRNAs are only separated by about 185 bp (Foury, 1989, 1990).

Vma4p (Subunit E). Vma4p is a peripheral 27-kDa subunit of the enzyme complex (Table IV). The function of the subunit is not known yet.

VMA5 and Vma5p (Subunit C)

VMA5. Beltrán *et al.* (1992) isolated the 42-kDa subunit from purified yeast vacuolar H^+-ATPase and determined its partial amino acid sequence. Based on this peptide information, an oligonucleotide was designed for screening clones containing *VMA5* from a yeast genomic DNA library. The nucleotide sequence of *VMA5* predicts a polypeptide of 373 amino acids with a calculated molecular mass of 42,287 daltons (Beltrán *et al.*, 1992). The protein is hydrophilic in nature with a neutral isoelectric point of 7.03. The predicted amino acid sequence contains the sequence of the 20 amino acids determined and shows 39% identity to the bovine counterpart in 311 overlapping amino acids. It has no homology to the γ subunit of the F_0F_1-ATPase as previously supposed (Nelson *et al.*, 1990).

Vma5p (Subunit C). Vma5p is a peripheral subunit of the enzyme complex and is liberated from the vacuolar membrane by cold inactivation (Noumi *et al.*, 1991). The function of the 42-kDa subunit C is not known yet.

VMA11 and Vma11p (Subunit *c′*)

VMA11. VMA11 was isolated from a yeast genomic DNA library by complementation of the *vma11* mutation (Umemoto *et al.*, 1991): the haploid strain NUY30 (*vma11 leu2*) was transformed with the DNA library on YEp13 (Yoshihisa and Anraku, 1989) and five colonies that grew on YP-glycerol

plates were isolated from about 12,000 Leu^+ transformants. These five positive transformants could also grow on YPD plates containing 100 mM $CaCl_2$. Two distinct plasmids have been recovered after the second round of transformation followed by testing for plasmid loss. The restriction maps of the two plasmids show that both inserts contain the same DNA fragment. The 1.8-kb *Eco*RV-*Spe*I fragment (pNUVA366) that complements the *vma11* mutation was identified after testing a series of deletions of the original 11-kb isolate pNUVA350, and it was confirmed that this complementing activity was not due to extragenic suppression (Umemoto *et al.*, 1991).

The nucleotide sequence of pNUVA366 shows that the authentic *VMA11* gene encodes a hydrophobic polypeptide of 164 amino acids with a calculated molecular mass of 17,037 daltons. It also contains the essential *Pvu*II site and a positive TATA sequence, TATA, 56 bp upstream of the first methionine codon in the open reading frame (Umemoto *et al.*, 1991).

The nucleotide sequence of the *VMA11* gene contains a nine-base repeat, AGCTGCCAT, at positions 72–80 and 99–107, which is not present in a reported sequence of the *TFP3* gene (Shih *et al.*, 1990) encoding a hydrophobic protein of 10 kDa. The deduced amino acid sequence shows extensive sequence identity (56.7% in 150 amino acids) to Vma3p (Umemoto *et al.*, 1991).

Vma11p (Subunit *c'*). Hirata and Anraku (1991)[4] demonstrated that Vma11p is located in the vacuolar membrane. Based on this finding and its extensive homology with Vma3p, the *VMA11* gene product is designated subunit *c'*.

The disruption of either the *VMA3* and *VMA11* gene causes loss of vacuolar acidification and leads to defective assembly of subunits A, B, and *c* of the H^+-ATPase (Umemoto *et al.*, 1991), suggesting that the functions of the two genes are independent. To confirm this particular point genetically, they constructed plasmids harboring each gene on the multicopy vector pYO325 and used these plasmids for analysis of multicopy suppression. Results indicated that *VMA11* and *VMA3* on multicopy plasmids do not suppress null mutations of *vma3* and *vma11*, respectively. Thus the two genes do not share the same function but function independently. Vma11p may be a second species of DCCD-binding proteolipid of the yeast vacuole because the deduced amino acid sequence predicts that a conserved glutamic acid residue of the DCCD-binding site in proteolipids of the F_0F_1-ATPase and the V-type ATPase is present in the sequence (Umemoto *et al.*, 1991).

The expression level of Vma11p seems much less than that of Vma3p. Interestingly, vacuolar membrane vesicles prepared from the *VMA11*-disrupted cell had lost Vma3p completely, and neither Vma1p nor Vma2p assembled on the membranes, although these two peripheral subunits were synthesized normally in the total cell extract. These results suggest that the function of Vma11p is a prerequisite for assembly of subunit *c* and then subunits A and B on the vacuolar membrane (Umemoto *et al.*, 1991).

VMA6, VMA12, and *VMA13*

VMA6 has been proposed to encode a 36 kDa-subunit of the H^+-ATPase in yeast (Bauerle *et al.*)[5] *VMA12* and *VMA13* have been shown to be the indispensable genes for expression of the enzyme activity (Ohya *et al.*, 1991). The nucleotide sequences of *VMA12* (Umemoto *et al.*)[6] and *VMA13* (Hirata *et al.*)[7] have been determined and suggest that Vma12p and Vma13p are a 25-kDa hydrophilic polypeptide with two membrane-spanning domains and a 54-kDa hydrophilic polypeptide with low homology for the γ subunit of *Sulfolobus acidocaldarius* (Denda *et al.*, 1990), repectively. Vma13p seems to be a counterpart of the 54-kDa subunit detected in the vacuolar H^+-ATPase from *Beta vulgarius* (Parry *et al.*, 1989).

ASSEMBLY *IN VIVO* OF THE H^+-ATPase SUBUNITS

A series of biochemical and immunological experiments have been carried out to study the assembly of the H^+-ATPase subunits onto the vacuolar membrane, using various null *vma* mutants (Kane *et al.*, 1991; Umemoto *et al.*, 1990, 1991). Only the potential function Vma11p in enzyme assembly is highlighted briefly in the preceding section. For an overview and current information on this subject readers are referred to the article by Patricia Kane, and Tom Stevens, in this volume.

[4]Hirata, R., and Anraku, Y., unpublished observations.

[5]Bauerle, C. M., Ho, M. N., Lindorfer, M. A., and Stevens, T. H., personal communication.

[6]Umemoto, N., Hirata, R., Ohya, Y., and Anraku, Y., unpublished observations.

[7]Hirata, R., Umemoto, N., Ohya, Y., and Anraku, Y., unpublished observations.

VACUOLAR MORPHOGENESIS IS A PREREQUISITE FOR EXPRESSION OF VACUOLAR FUNCTION

Current studies from our laboratory have demonstrated that the yeast vacuole is the center for regulation of ionic homeostasis in the cytosol (Anraku *et al.*, 1989, 1991a,b). Even if the *VMA* genes are all expressed normally, the large volume of a central vacuole is needed physiologically to confer the organelle with a high capacity for maintenance of homeostatic levels of the cytosolic free Ca^{2+} and basic amino acids (Kitamoto *et al.*, 1988a,b; Ohsumi *et al.*, 1988) and for compartmentation of a number of vacuolar proteases (Banta *et al.*, 1990; Wada *et al.*, 1990). Wada *et al.* (1990) have developed several genetic methods for isolating yeast mutants that are defective in vacuolar morphogenesis and identified genes that are involved in acquisition of large vacuoles. Interestingly, several mutations in the *VAM* genes (for *va*cuolar *m*orphology), which result in complete loss of central vacuoles, show the Ca^{2+}-sensitive phenotype of type I *cls* mutations (Ohya *et al.*, 1986, 1991) and are allelic to respective *vps*, *pep*, and *end* mutations for low vacuolar peptidases and missorting of carboxypeptidase Y (see Banta *et al.*, 1990; Wada *et al.*, 1990). These results suggest that a recessive mutation on a single chromosomal gene can cause pleiotropic defects in vacuolar lytic function and vacuolar morphogenesis.[8]

CONCLUSION AND PERSPECTIVES

We have summarized the present status of genetic information on how many *VMA* genes are required for full expression and regulation of the yeast vacuolar H^+-ATPase. Nine *VMA* genes have proven to be essential for expression of the enzyme activity. The unique structures of the *VMA* genes are discussed, in addition to the phenotypes of the null *vma* mutations.

The enzyme is truly a large heterooligomeric complex, and the nature of the subunit composition and function awaits further elucidation. Cell biological issues regarding the biogenesis of the holoenzyme and vacuolar morphogenesis remain to be studied and concern the next immediate question of interest: how this V-type H^+-ATPase can accomplish vacuolar acidification and control homeodynamic chemiosmosis in a eukaryotic cell system.

[8]Wada, Y., Ohsumi, Y., and Anraku, Y., *J. Biol. Chem.*, in press.

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