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MOLECULAR GENETICS OF THE YEAST VACUOLAR H^+-ATPase

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Summary

The yeast vacuolar proton-translocating ATPase was discovered in 1981 as the first member of the V-ATPases, which are now known to be ubiquitously distributed in eukaryotic vacuo-lysosomal organelles and archaebacteria. Nine *VMA* genes that are indispensable for expression of vacuolar ATPase activity have been identified in the yeast *Saccharomyces cerevisiae*. *VMA1*, *VMA2*, *VMA3*, *VMA5* and *VMA6* were cloned and characterized on the basis of partial amino acid sequences determined with the purified subunits. Genetic and biochemical studies of the yeast Pet$^-$*cls* mutants have demonstrated that they are related to *vma* defects. Based on this evidence, *VMA11* (*CLS9*), *VMA12* (*CLS10*) and *VMA13* (*CLS11*) were isolated from a yeast genomic DNA library by complementation of the *vma11*, *vma12* and *vma13* mutations, respectively. This article summarizes currently available information on the *VMA* genes and the molecular biological functions of the *VMA* gene products.

Introduction

The fungal vacuole is an acidic compartment which plays essential roles in metabolic storage and in cytosolic ion and pH homeostasis. In addition, it functions in endolytic macromolecular degradation in a manner similar to that occurring in phagocytotic animal lysosomes (Figs 1 and 2: Anraku, 1987*a*,*b*; Anraku *et al.* 1989; Klionsky *et al.* 1990). During the last 10 years, it has become known that a new, distinct class of H^+-pumping ATPase, a V-ATPase, exists ubiquitously in vacuo-lysosomal and endomembranous organelles, including fungal and plant vacuoles, animal lysosomes, coated vesicles, Golgi bodies, chromaffin granules and synaptic membrane vesicles (for recent reviews, see Anraku *et al.* 1989, 1991*a*, 1992; Forgac, 1989; Nelson and Taiz, 1989; Stone *et al.* 1989). The V-ATPase is also present in plasma membranes of vertebrate renal tissues (Gluck, 1992), osteoclasts (Chatterjee *et al.* 1992) and insect gastrointestinal and sensory epithelia (Harvey, 1992; Klein, 1992; Wieczorek, 1992).

Biochemical studies of yeast vacuoles originated with the work of Ohsumi and Anraku (1981), who established a simple method for separating intact vacuoles of high purity from *Saccharomyces cerevisiae*. Kakinuma *et al.* (1981) found that a preparation of vacuolar membrane vesicles with a right-side-out orientation had an unmasked Mg^{2+}-ATPase activity with an optimal pH of 7.0. The activity was sensitive to

Key words: V-ATPase, *VMA* genes, *Saccharomyces cerevisiae*, vacuole.

dicyclohexylcarbodiimide (DCCD) and was stimulated threefold by the protonophore uncoupler SF6847 and 1.5-fold by the H^+/K^+ antiporter nigericin. ATP-hydrolysis-dependent uptake of protons into vacuolar membrane vesicles has been demonstrated directly by the change in quenching of 9-aminoacridine and quinacrine fluorescence (Kakinuma *et al.* 1981; Ohsumi and Anraku, 1981). The electrochemical potential difference of protons across the vacuolar membrane generated upon ATP hydrolysis was determined to be 180 mV, consisting of a proton gradient of 1.7 pH units, interior acid, and of a membrane potential of 75 mV, interior positive (Kakinuma *et al.* 1981).

Studies from our laboratory have shown that the vacuolar membrane of yeast is equipped with two distinct Cl^- transport systems, each of which contributes to the formation of a chemical gradient of protons across the vacuolar membrane by shunting the membrane potential generated by the H^+-ATPase (Fig. 3: Anraku *et al.* 1989, 1992; Wada *et al.* 1992*a*). Vacuolar acidification is a prerequisite for operation of amino acid/H^+antiporters (Ohsumi and Anraku, 1981; Sato *et al.* 1984*a*,*b*), a Ca^{2+}/H^+antiporter (Ohsumi and Anraku, 1983) and a K^+ channel (Wada *et al.* 1987; Tanifuji *et al.* 1988). In cases where this ability to acidify is lost, vacuolar protein transport and nonspecific fluid-phase endocytosis are markedly affected (Klionsky *et al.* 1990; Mellman *et al.* 1986; Umemoto *et al.* 1990; Yamashiro *et al.* 1990).

Vacuolar H^+-ATPases are large multimeric enzymes with a functional relative molecular mass (M_r) of about 500×10^3 (Bowman *et al.* 1986; Hirata *et al.* 1989) and contain at least nine subunits (Adachi *et al.* 1990; Arai *et al.* 1988; Bowman *et al.* 1989; Kane *et al.* 1989; Moriyama and Futai, 1990; Moriyama and Nelson, 1987*a*,*b*; Parry *et al.* 1989; Xie and Stone, 1986). The enzymes are sensitive to bafilomycin A_1 (Bowman *et al.* 1988*b*; Umemoto *et al.* 1990; Yoshimori *et al.* 1991). The proposed reaction mechanism (Hirata *et al.* 1989; Uchida *et al.* 1988) is similar to that for mitochondrial and bacterial F_1F_o-ATPases (see Futai *et al.* 1988, 1992).

Taiz and his coworkers first cloned and sequenced a cDNA encoding the carrot 69×10^3 M_r polypeptide, a catalytic subunit of the enzyme (Zimniak *et al.* 1988). Then, Bowman *et al.* (1988*a*,*c*) reported isolation and sequencing of two genes from *Neurospora crassa*, *vma1* and *vma2*, which they designated for *v*acuolar *m*embrane *A*TPase. These earlier contributions have provided breakthroughs for molecular biological and genetic studies of V-ATPases.

This article addresses genetic and molecular biological views of the yeast vacuolar H^+-ATPase, emphasizing the manipulation of genetic screening for mutations with defective

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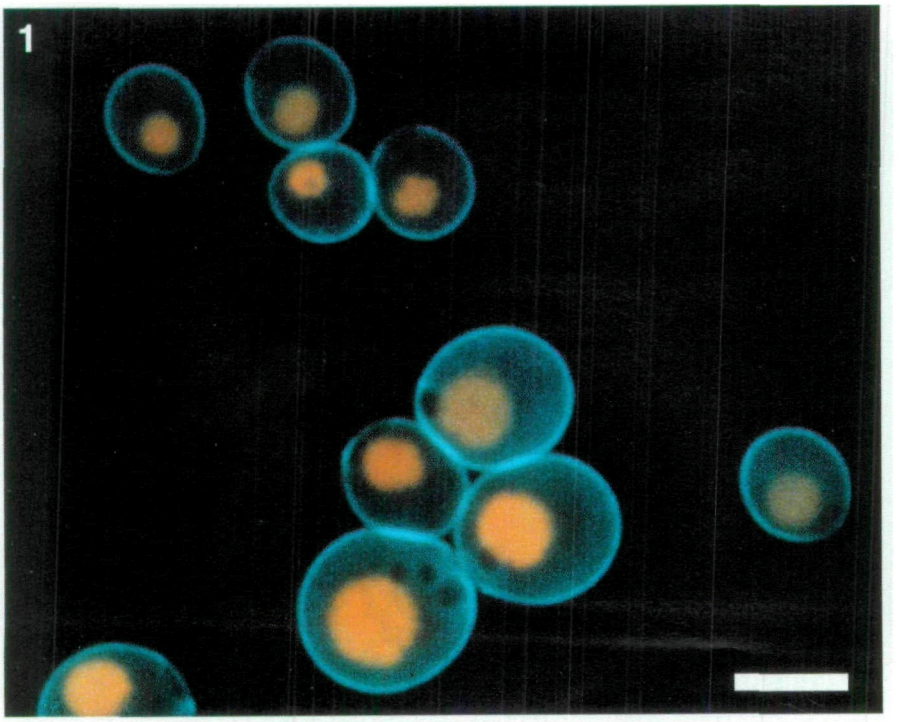
Fig. 1. Localization of *ade* fluorescence in the central vacuole (from Y. Wada, Y. Ohsumi and Y. Anraku, unpublished observations). Haploid cells YW1-6A (MATα *ade1* Vam$^+$) were stained with 0.2% Aniline Blue WS and observed under a fluorescence microscope (Photomicroscope III, Zeiss) with G405 and LP495 for excitation and barrier filters, respectively. The photograph was taken with Ektachrome 400 film. Scale bar, 3 μm.

Fig. 2. Internalization of Lucifer Yellow CH in vacuoles (from Y. Wada, Y. Ohsumi and Y. Anraku, unpublished observations). Diploid cells YW1–7A/A2–1–1A (Vam$^+$) were incubated with Lucifer Yellow CH (10 mg ml^{-1}) for 1 h at 30°C followed by incubation with 0.05% Aniline Blue WS and 2% glucose. The vacuoles and cell wall were observed as in Fig. 1. The photograph was taken with TMAX-400 film. Scale bar, 3 μm.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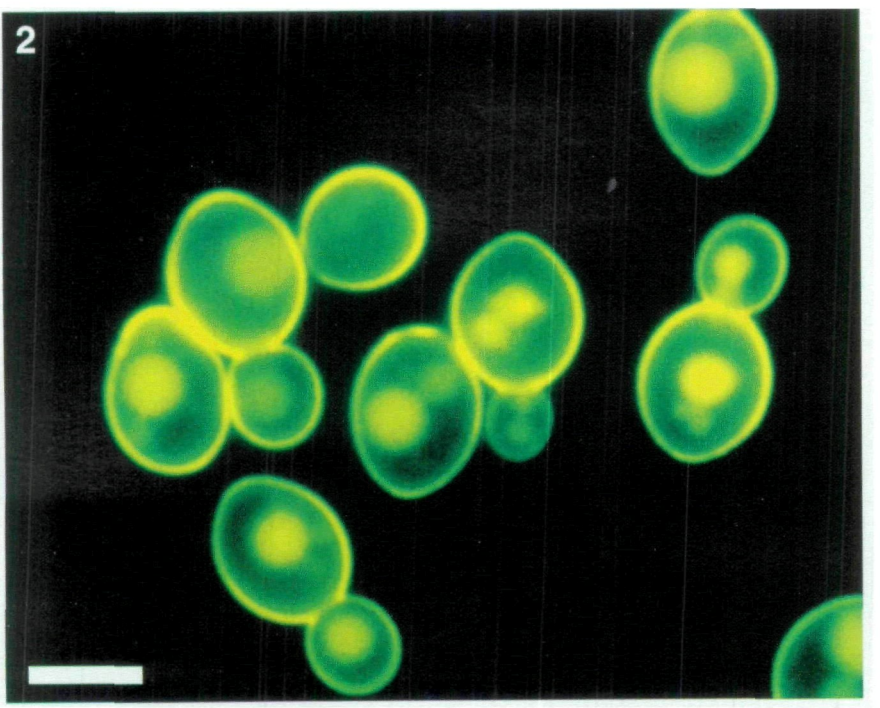
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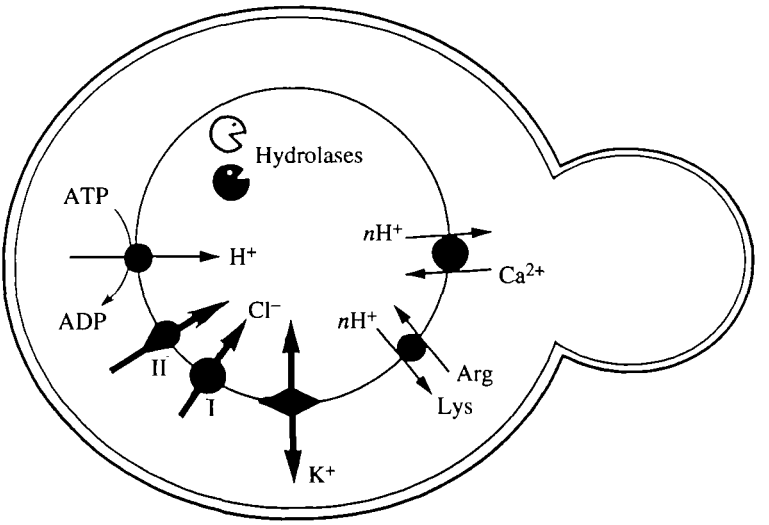

Fig. 3. The vacuolar world in *Saccharomyces cerevisiae*. The vacuole contains a number of proteases and other hydrolases for endolytic macromolecular degradation. The vacuolar membrane is equipped with various primary and secondary chemiosmotic transport systems. The H^+-ATPase as a primary H^+ pump energizes the membrane and causes an electrochemical gradient of protons, which drives the antiporters for amino acids and Ca^{2+}, and the K^+-channel. Chloride transport systems I and II are DIDS-insensitive and DIDS-sensitive, respectively. See text for details.

vacuolar acidification and, hence, of the *VMA* genes that affect expression of the enzyme activity in *Saccharomyces cerevisiae*.

Characterization of *VMA* genes

Initially, the yeast vacuolar H^+-ATPase in *S. cerevisiae* was partially purified and characterized as a three-subunit enzyme (Uchida *et al.* 1985). Kane *et al.* (1989) examined the original method of purification more carefully and demonstrated that the fraction with the highest specific activity included eight polypeptides with apparent M_r values of 100, 69, 60, 42, 36, 32, 27 and 17×10^3. They also showed that a monoclonal antibody raised against the 69×10^3 M_r polypeptide immunoprecipitated this eight-subunit enzyme, suggesting that all eight polypeptides are good candidates for being subunits of the enzyme.

Based on information from the peptide and nucleotide sequences of respective subunits and cDNAs 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.* 1991*a*; 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 42×10^3 M_r subunits, respectively. The sequence of *VMA2* (Nelson *et al.* 1989) was determined by using a synthetic oligonucleotide derived from the counterpart cDNA (Manolson *et al.* 1988). *VMA4* was accidentally discovered and characterized during a sequence study of *MIP1* (Foury, 1990).

Growth phenotypes of *vma* mutants

Anraku and coworkers (Hirata *et al.* 1990; Ohya *et al.* 1991; Umemoto *et al.* 1990) have studied growth phenotypes of the chromosomal *VMA1*-, *VMA2*- and *VMA3*-disrupted mutants. The three mutants can grow well in YPD medium (2% Bacto-yeast extract, 2% polypeptone and 2% glucose: Ohya *et al.* 1991), indicating that each *VMA* gene is not indispensable for growth. However, they all show a Pet$^-$*cls* phenotype (Ohya *et al.* 1991): the *vma* null mutants cannot grow on a YPD plate containing 100 mmol l^{-1} $CaCl_2$ and on YP plates (2% Bacto-yeast extract, 2% polypeptone and 2% agar: Ohya *et al.* 1991) containing nonfermentable carbon sources such as 3% glycerol and 2% succinate. The Pet$^-$ phenotype was unexpected and difficult to explain at this stage of study, but the calcium-sensitive *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* (Ohya *et al.* 1991).

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 a *pet* mutant and this Pet$^-$ phenotype co-segregates with the Cls$^-$ phenotype (Ohya *et al.* 1986). A genetic study was 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* 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; DCCD-sensitive ATPase activity and ATP-dependent activity for Ca^{2+} uptake were not detected in these vesicles (Ohya *et al.* 1991). Based on these genetic and cell biological data, it was concluded that the Pet$^-$*cls* mutants were ascribable to *vma* defects. Thus, *CLS9*, *CLS10* and *CLS11* are a family of *VMA* genes and are designated henceforth *VMA11*, *VMA12* and *VMA13*, respectively (Ohya *et al.* 1991).

In yeast cells growing in YPD medium, the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is critically regulated at about 150–180 nmol l^{-1} (Iida *et al.* 1990*a*,*b*). Measurements of $[Ca^{2+}]_i$ in individual cells of the Pet$^-$*cls* mutants yielded a mean value of 900–1100 nmol l^{-1}, as a primary consequence of the *vma* mutation (Ohya *et al.* 1991). Thus, the sixfold increase in $[Ca^{2+}]_i$ may trigger serious metabolic perturbation and is injurious to growth of yeast cells (Anraku *et al.* 1991*b*; Galons *et al.* 1990). 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). The *vma* mutants show pH-conditional growth phenotypes (Umemoto *et al.* 1991). These pH-conditional growth phenotypes have become known (Beltrán *et al.* 1992; Noumi *et al.* 1991; Yamashiro *et al.* 1990) and can be used for selecting new genes of the *VMA* family.

Table 1. *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		Other name used
		Calculated[a]	Apparent[b]	
I. Gene encoding a peripheral polypeptide				
VMA1[c]	*A*	67.7	69	*TFP1*
VMA2[d]	*B*	57.7	60	*VAT2*
VMA13[e]		54.4		
VMA5[f]	*C*	42.3	42	*VAT5*
VMA6[g]	*D*	36		
VMA4[h]	*E*	26.6	27	
II. Gene encoding an integral polypeptide				
VMA3[i]	*c*	16.4	17	*TFP3*
VMA11[j]	*c'*	17.0		
VMA12[k]		25.3		
III. Gene not identified yet				
			100	
			32	

[a]Molecular mass (kDa) is calculated from the deduced amino acid sequence of the respective gene.
[b]Relative molecular mass ($\times 10^{-3}$) estimated from SDS–polyacrylamide gel electrophoresis; (see Kane *et al.* (1989).
[c]Hirata *et al.* (1990); Shih *et al.* (1988).
[d]Anraku *et al.* (1991*a*); Nelson *et al.* (1989); Yamashiro *et al.* (1990).
[e]Ohya *et al.* (1991); R. Hirata, N. Umemoto, Y. Ohya and Y. Anraku (unpublished data).
[f]Beltrán *et al.* (1992).
[g]C. M. Bauerle, M. N. Ho, M. A. Lindorfer and T. H. Stevens (personal communication).
[h]Foury, (1990).
[i]Anraku *et al.* (1991*a*); Nelson and Nelson, (1989); Umemoto *et al.* (1990).
[j]Ohya *et al.* (1991); Shih *et al.* (1990); Umemoto *et al.* (1991).
[k]Ohya *et al.* (1991); N. Umemoto, R. Hirata, Y. Ohya and Y. Anraku (unpublished data).

Structure and function of the *VMA* gene products

By 1991, nine *VMA* genes had been identified from the yeast *S. cerevisiae* (Table1). Table 1 also lists the subunits encoded by the respective genes, with their names revised according to the proposals of Anraku *et al.* (1992) and of Nelson and Taiz (1989). Subunits designated by an italic capital letter are polypeptides that are peripheral in nature and are a counterpart of F_1 of the ATP synthase, whereas those designated by an italic lower case are subunits that are integral in nature and are a counterpart of the F_o sector. All the candidate subunits detected biochemically and immunochemically (Kane *et al.* 1989) are listed for reference.

VMA1 *and Vma1p (subunit* A*)*

VMA1 was isolated from a yeast genomic DNA library (Yoshihisa and Anraku, 1989) by hybridization with a 39-mer oligonucleotide probe corresponding to the 13 amino acid

sequence in the purified 67×10^3 M_r subunit (Hirata *et al.* 1990). The nucleotide sequence of the gene predicts a polypeptide of 1071 amino acids (118 635 Da), which is much larger than the mature form of the 67×10^3 M_r 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* (69×10^3 M_r) (Zimniak *et al.* 1988) and *Neurospora crassa* (67×10^3 M_r) (Bowman *et al.* 1988*c*). 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.* 1991*a*; 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 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 is 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.* 1991). Recently, Kane *et al.* (1990) have shown that yeast cells carrying *VMA1* under control of the inducible *GAL10* promoter express a 119×10^3 M_r polypeptide of the unprocessed *VMA1* gene product in galactose medium and that the precursor undergoes post-translational cleavage and splicing to yield the mature 67×10^3 M_r subunit *A* and a 50×10^3 M_r polypeptide.

Assuming that the whole stretch of the nonhomologous insert (residues 285–738) is removed from the *VMA1* product, a molecular mass of 67 722 Da is calculated for the mature subunit consisting of 617 amino acids. This is in good agreement with the value for the relative molecular mass of 67×10^3 M_r estimated by SDS–polyacrylamide gel electrophoresis (Hirata *et al.* 1990). Thus, the deduced primary sequence of yeast Vma1p is very similar to those of the *Neurospora crassa* (Bowman *et al.* 1988*c*) and *Daucus carota* (Zimniak *et al.* 1988) counterparts: About 73 and 60%, respectively, of the residues are identical with the fungal and plant sequences.

Vma1p is the catalytic subunit of the enzyme complex (Uchida *et al.* 1988) and localizes to the cytoplasmic side of the vacuolar membrane (Fig. 4). Consistent with this biochemical evidence, the deduced primary sequence of Vma1p shows about 25% sequence identity over 400 residues with β subunits of F_1F_o-ATPases (Hirata *et al.* 1990). Vma1p has consensus sequences for the nucleotide-binding domain proposed by Walker *et al.* (1982) and contains conserved amino acid residues that have proved to be important for ATP hydrolysis (Futai *et al.* 1989), suggesting that the catalytic subunits from the two classes of ATPases share similar structures and mechanisms of ATP hydrolysis.

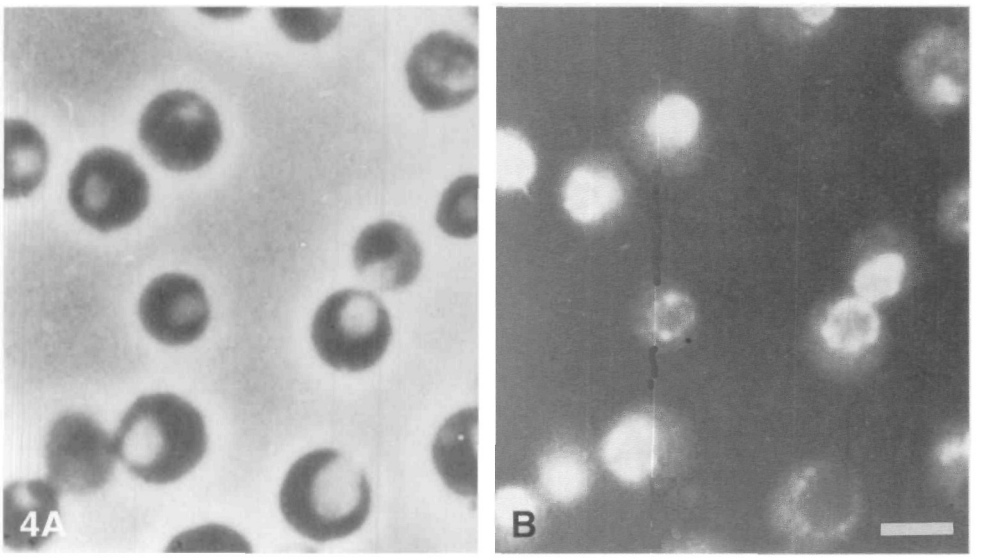

Fig. 4. Immunofluorescence detection of the H^+-ATPase on yeast vacuoles (Umemoto *et al.* 1990). Spheroplasts of haploid strain X2180-1A were subjected to indirect immunofluorescence staining with the monoclonal antibody R70 raised against subunit *A* of the H^+-ATPase. (A) Cell morphology examined by phase-contrast photomicrography. (B) Fluorescence microscopic view of the same cells, showing distinct localization of the enzyme on the membrane of the central vacuole. Scale bar, 3 μm.

VMA2 *and Vma2p (subunit* B)

Based on the nucleotide sequence information of the 57×10^3 M_r subunit of *Arabidopsis thaliana* vacuolar H^+-ATPase (Manolson *et al.* 1988), a cDNA clone encoding a counterpart subunit in yeast has been isolated (Nelson *et al.* 1989; Yamashiro *et al.* 1990). The predicted amino acid sequence deduced from the nucleotide sequence proved to contain all the four peptides that were determined with the purified 57×10^3 M_r subunit from *S. cerevisiae* (Anraku *et al.* 1991*a*; 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 57×10^3 M_r subunit in the cell lysate.

The nucleotide sequence of *VMA2* predicts a polypeptide of 517 amino acids (57 749 Da). Comparison of sequence homology (Yamashiro *et al.* 1990) revealed extensive sequence identities of 82, 74, 54, 58 and 74%, respectively, to the 60×10^3 M_r subunits from *Neurospora crassa* (Bowman *et al.* 1988*a*), *Arabidopsis thaliana* (Manolson *et al.* 1988), *Sulfolobus acidcaldarius* (Denda *et al.* 1988*a*,*b*), *Methanosarcia barkeri* (Inatomi *et al.* 1989) and human endomembrane (Südhof *et al.* 1989).

Vma2p seems to be present in an equimolar amount with Vma1p in purified enzymes from yeast (Uchida *et al.* 1985; Kane *et al.* 1989). Vacuoles isolated from the *vma2* cells showed no vacuolar H^+-ATPase activity and no vacuolar acidification ability (Ohya *et al.* 1991; Yamashiro *et al.* 1990), so this major subunit is essential for the expression of enzyme activity, probably functioning as a regulatory component (Hirata *et al.* 1989).

VMA3 *and Vma3p (subunit* c)

Two independent strategies were adopted for cloning the *VMA3* gene. For a hybridization probe of *VMA3* from a yeast genomic DNA library, Nelson and Nelson (1989) synthesized a 105-mer oligonucleotide based upon 35 amino acids of the C terminus of the $17\times10^3 M_r$ 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 upon the determination of the N-terminal 17 amino acids with the purified $16\times10^3 M_r$ proteolipid from yeast vacuoles. Nucleotide sequencing of all the candidates revealed that they contain a single open reading frame encoding a hydrophobic polypeptide of 160 amino acids (16350 Da) (Nelson and Nelson, 1989; Umemoto *et al.* 1990). *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 $17\times10^3 M_r$ 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). The C-terminal half of yeast Vma3p showed significant homology (about 35% identity) to $8\times10^3 M_r$ 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 was less marked and showed about 27% identity to $8\times10^3 M_r$ proteolipids of thermophilic bacterium PS3 and *Bacillus megaterium* (Brusilow *et al.* 1989; Sebald and Hoppe, 1981). This suggests that the yeast *VMA3* gene is a duplicated and diverged form of the genes encoding $8\times10^3 M_r$ proteolipids of the F_o sectors in F_1F_o-ATPases (Nelson and Nelson, 1989).

Subunit *c* in the partially purified yeast enzyme bound DCCD (Uchida *et al.* 1985), suggesting that it may function as a part of a channel for proton translocation in the H^+-ATPase complex (Kakinuma *et al.* 1981). Hydropathy analysis 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_1F_o-ATPases.

VMA4 *and Vma4p (subunit* E)

Foury (1990) discovered *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) was determined; it predicted a hydrophilic polypeptide of molecular mass 26.6 kDa. The deduced amino acid sequence shows 34% identity to the $31\times10^3 M_r$ subunit of the V-ATPase from kidney microsomes (Hirsch *et al.* 1988). *VMA4* and *MIP1* were found to be located on chromosome XV and the initiation sites of their mRNAs are only separated by about 185 bp (Foury, 1989, 1990). Vma4p is a peripheral $27\times10^3 M_r$ subunit of the enzyme complex (Table 1). The function of the subunit is not known yet.

VMA5 *and Vma5p (subunit* C)

Beltrán *et al.* (1992) isolated the 42×10^3 M_r 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 (42 287 Da). 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. Vma5p is a peripheral subunit of the enzyme complex and is liberated from the vacuolar membrane by sodium carbonate treatment (Kane *et al.* 1992). The function of the 42×10^3 M_r subunit *C* is not known yet.

VMA11 *and Vma11p (subunit* c′)

VMA11 was isolated from a yeast genomic DNA library by complementation of the *vma11* mutation (Umemoto *et al.* 1991): a haploid strain NUY30 (*vma11 leu2*) was transformed with the DNA library on YEp13, 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 a YPD plate containing 100 mmol l^{-1} $CaCl_2$. Two plasmids were recovered after the second round of transformation followed by tests of 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 as a minimal essential region was restricted after testing a series of deletions of the inserts constructed from the 11-kb original isolate pNUVA350, confirming that this complementing activity was not due to extragenic suppression by integrative mapping with this clone (Umemoto *et al.* 1991). The nucleotide sequence of pNUVA366 shows that the authentic *VMA11* gene encodes a hydrophobic polypeptide of 164 amino acids (17 037 Da).

The nucleotide sequence of the *VMA11* gene contains a nine-base repeat, AGCTGCCAT, at positions 72–80 and 99–107; these sequences were not present in a reported sequence of the *TFP3* gene (Shih *et al.* 1990) encoding a hydrophobic protein of 10×10^3 M_r. The deduced amino acid sequence predicts a surprising coincidence in amino acid composition with Vma3p, showing extensive sequence identity (56.7% in 150 amino acids) to Vma3p (Umemoto *et al.* 1991). R. Hirata and Y. Anraku (unpublished observations) demonstrated that Vma11p is located in the vacuolar membrane. Based on this finding and its extensive homology with Vma3p, the *VMA11* gene product was designated as subunit *c′* (Table 1).

The disruption of either one of the *VMA3* and *VMA11* genes causes loss of vacuolar acidification (Fig. 5) 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 point genetically, they constructed plasmids harboring each gene on multicopy vector pYO325 and used them 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 functions, but function independently. Vma11p may be a second species of DCCD-binding

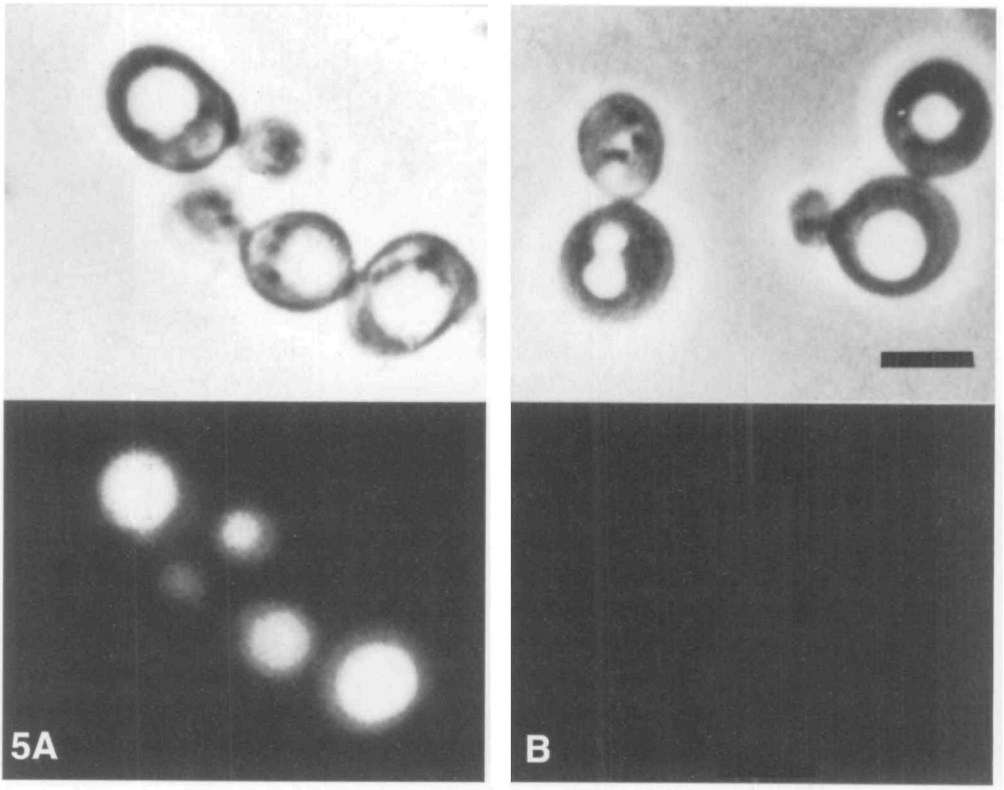

Fig. 5. Quinacrine accumulation in vacuoles (Umemoto *et al.* 1990). Phase contrast (upper) and fluorescence (lower) images of cells stained with the weakly basic dye quinacrine. (A) Wild-type cells; (B) *vma3* mutant cells. Scale bar, 3 μm. Central vacuoles are seen as a bright area in the cells. The *vma3* mutant cells (NUY29H1) have morphologically normal vacuoles but the vacuoles cannot accumulate quinacrine because of the defect of ΔpH formation.

proteolipid from 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_1F_o-ATPase and the V-ATPase is present in the sequence (Umemoto *et al.* 1991).

Vacuolar membrane vesicles prepared from the *VMA11*-disrupted cells had lost Vma3p completely, and neither Vma1p nor Vma2p assembled on the membranes, although these two peripheral subunits were synthesized normally and were present 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

It has been proposed that *VMA6* encodes a $36\times10^3 M_r$ subunit of the H^+-ATPase in yeast (C. M. Bauerle, M. N. Ho, M. A. Lindorfer and T. H. Stevens, personal communication). *VMA12* and *VMA13* have been shown to be indispensable genes for expression of the enzyme activity (Ohya *et al.* 1991). The nucleotide sequences of

VMA12 (N. Umemoto, R. Hirata, Y. Ohya and Y. Anraku, unpublished data) and *VMA13* (R. Hirata, N. Umemoto, Y. Ohya and Y. Anraku, unpublished data) have been determined; the sequences predict that Vma12p is a 25×10^3 M_r integral polypeptide with two membrane-spanning domains and that Vma13p is a 54×10^3 M_r hydrophilic polypeptide with low homology for the γ subunit of *Sulfolobus acidocaldarius* (Denda *et al.* 1990). Vma13p seems to be a counterpart of the 54×10^3 M_r subunit detected in the vacuolar H^+-ATPase from *Beta vulgarius* (Parry *et al.* 1989).

Vacuolar morphogenesis is a prerequisite for expression of vacuolar function

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, 1991*a,b*, 1992). Even if a family of the *VMA* genes is all present and normal, the large volume of a central vacuole is needed physiologically to confer on the organelle a high capacity for maintenance of homeostatic levels of cytosolic free Ca^{2+} and basic amino acids (Kitamoto *et al.* 1988*a,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, 1992*b*) have developed several genetic methods for isolating yeast mutants defective in vacuolar morphogenesis and they have identified genes involved in the acquisition of large vacuoles. Interestingly, several mutations in the *VAM* genes (*vam1*, *vam5*, *vam8* and *vam9*; for *va*cuolar *m*orphology), which result in complete loss of central vacuoles (Wada *et al.* 1992*b*), show a Ca^{2+}-sensitive phenotype of type-I *cls* mutation (Ohya *et al.* 1986, 1991) and are allelic to the respective *vps*, *pep* and *end* mutations for low vacuolar peptidases and missorting of carboxypeptidase Y. These results suggest that a recessive mutation on a single chromosomal gene can cause pleiotropic defects in vacuolar lytic function and vacuolar morphogenesis (Wada *et al.* 1992*b*).

Conclusion and perspectives

This article summarizes 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 proved to be essential for expression of the enzyme activity. The structure and function of the *VMA* gene products are discussed, in addition to the phenotypes of the null *vma* mutations.

The enzyme is a large hetero-oligomeric complex with at least nine subunits but the nature of subunit composition and function awaits further elucidation. Molecular biological issues regarding the biogenesis of this holoenzyme and the vacuolar morphogenesis remain to be studied. Of great current interest is the mechanism by which this typical V-type H^+-ATPase can accomplish vacuolar acidification and control homeodynamic chemiosmosis in a eukaryotic cell system.

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