Molecular Characterization of VAC1, a Gene Required for Vacuole Inheritance and Vacuole Protein Sorting*

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Cell division requires an accurate partitioning of cytoplasmic organelles. The segregation of vacuoles in the budding yeast *Saccharomyces cerevisiae* occurs at a specific time in the cell cycle and is spatially targeted to the small bud. Several yeast vac mutants have been isolated which are defective in this process. We have now cloned the VAC1 gene, corresponding to the first of these mutants, vac1-1. This gene encodes a protein of 515 amino acids, without homolog in the current data bases. It contains neither long hydrophobic stretches nor a classical leader peptide. The most notable aspect of the sequence is the presence of three zinc fingers. Yeast in which the VAC1 gene has been entirely deleted are viable. However, they grow more slowly than wild-type cells and only form microcolonies when grown on glycerol at 37 °C. These yeast are defective in vacuole segregation at both the permissive and nonpermissive temperatures. The vac1 mutant was previously shown to mislocalize carboxypeptidase Y to the cell surface, suggesting that Vac1p is involved in more than one vesicular traffic pathway.

While cytoplasmic organelles with high copy number may segregate by random diffusion at cytokinesis (Birky, 1983), organelles with low copy number must employ specific mechanisms to ensure their accurate partitioning into daughter cells. No single mechanism accounts for the segregation of each type of organelle. For example, mammalian Golgi divide by synchronized vesiculation, segregation of the vesicles, and re-fusion in the daughter cells to form the characteristic stacked cisternae (Warren, 1985). In contrast, the yeast nuclear membrane remains intact during mitosis and divides by septation.

The yeast vacuole partitions by a very different mechanism (Weisman et al., 1987; Weisman and Wickner, 1988). Early in S phase, shortly after bud emergence, the new vacuole is founded in the bud. Studies with zygotes (Weisman and Wickner, 1988) bearing the endogenous ade2 fluorophore (Weisman et al., 1987) in one parental vacuole demonstrated that this new bud vacuole is founded by a highly directed stream of membrane-limited structures. Using a variety of fluorophores, these structures appear as tubules or vesicles (Weisman and Wickner, 1988; Weisman et al., 1990; Raymond et al., 1990; de Mesquita et al., 1991). These structures appear soon after bud emergence and persist for approximately 20 min (de Mesquita et al., 1991). Thus, vacuole inheritance is both cell cycle-specific and highly spatially directed.

We have isolated several yeast strains which are defective in this process (Weisman et al., 1990; Shaw and Wickner, 1991). In these vac mutants, the bud grows to normal size and at cytokinesis has a normal content of mitochondria and nuclear DNA, yet has little or no vacuolar material (Weisman et al., 1990; Shaw and Wickner, 1991). The majority of these mutants are defective in both vacuole segregation as well as targeting newly made proteins from the Golgi to the vacuole. In this paper, we report the further characterization of the first of these mutants to be identified, vac1-1. We have used this mutant to clone and sequence the wild-type gene. VAC1 encodes a protein with three zinc fingers which is otherwise without homolog in current data bases. We propose that Vac1p is either a peripheral membrane protein or cytoskeletal protein which functions in several interorganellar traffic pathways.

**MATERIALS AND METHODS**

**Strains, Plasmids, and Cell Culture—*Escherichia coli* DH5α was used for amplification of all plasmids, except where cleavage with the restriction enzyme BclI was required. In this case, plasmids were transformed into *E. coli* GM2163, a dam− strain.**

The VAC1 gene was cloned by transforming LWY148, vac1-1, ura3-52, ade2-102 (Weisman et al., 1990) with a yeast multicopy genomic library (Carlson and Botstein, 1982) generously provided by Dr. David Botstein (Stanford). The isogenic VAC1 strain LWY147, ura3-52, ade2-102 was utilized to map the original chromosomal location of the complementing VAC1 clone relative to vac1-1. Construction of LWY150 MATα, leu2-3,112, his4-519 in which the VAC1 gene is replaced with TRP1 is described below. LWY151, MATα, trp1-289 is the VAC1 sister spore from the same tetrad and served as the wild-type control. Yeast were grown on yeast extract/peptone/dextrose medium (Rose et al., 1990) (YPD). Yeast transformed with DNA containing either TRP1 or URA3 were selected on synthetic dextrose minimal medium, supplemented as described but lacking either tryptophan or uracil, respectively (Rose et al., 1990).

**PCR and Synthesis of Oligonucleotide Probes—For both VAC1 deletion constructs, the vector pLW101, comprised of the yeast shuttle vector pRS316 (Sikorski and Hieter, 1989) and 8 kb of *VAC1* DNA, was used. Restriction enzyme sites were placed at the ends of the *URA3* DNA fragment and the *TRP1* fragment by means of PCR and Synthesis of Oligonucleotide Probes—For both VAC1 deletion constructs, the vector pLW101, comprised of the yeast shuttle vector pRS316 (Sikorski and Hieter, 1989) and 8 kb of *VAC1* DNA, was used. Restriction enzyme sites were placed at the ends of the *URA3* DNA fragment and the *TRP1* fragment by means of polymerase chain reaction (Higuchi, 1989). Amplification with 1.5 units of Taq polymerase (Perkin-Elmer Cetus) was performed at 53 °C in 20 μl of 50 mM KCl, 100 mM TrisCl, pH 8.4, 100 mM MgCl2, 1 mg/ml bovine serum albumin with 5 x 10−6 pmol of vector containing *URA3* or *TRP1*, 2 mM dATP, 2 mM dCTP, 2 mM dTTP, 2 mM dGTP, and 20 pmol of each primer. For the *URA3* construct, the oligonucleotides 5′-CCCGAATTCATGTTTGACAGCTTAT-3′ and 5′-CCCCGTCACCGCATTAAGACGTTTTCCTTTC-3′ were synthesized. The 1.2-kb fragment obtained from the polymerase chain reaction synthesis was digested with BstEII and

1 The abbreviations used are: kb, kilobase pair(s); FITC, fluorescein isothiocyanate.

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EcoRI, purified on a 0.7% agarose gel, and directly ligated into the 11-kb fragment from the plW101 vector which had been digested to completion with BstEII and partially digested with EcoRI. For the TRP1 construct, the oligonucleotides 5' -CCCTGAGCCGAGTAAACCTATGGCCACGGC-3' and 5' -CCCGGTCAGCCGTTCCTAAGGAGCGC-3' were used as primers. In this case, the 0.9-kb fragment was first subcloned into pCR1000 (Invitrogen), the resulting plasmid was amplified in E. coli GM2163, and the TRP1 fragment released by digestion with BclI and BstEII. After purification of the 0.9-kb fragment on a 0.7% agarose gel, it was ligated into the 10.5-kb fragment of plW101 which had been digested to completion with BstEII and partially digested with BclI.

**Sequencing Strategy**—Sequencing and complementation analysis were performed on two sets of nested deletions. The 5-kb SacI-SacII and the 3-kb SacII-SpeI fragments were subcloned into the polylinker of pRS316 (Sikorski and Hieter, 1989), a yeast shuttle vector which was generously provided by Dr. Philip Hieter (Johns Hopkins University). The 3-kb insert was used for the creation of nested deletions starting at the SacII site, whereas the deletions of the 3-kb insert originated from the SpeI site. The respective clones were cut in the polylinker with KpnI and HindIII, and limited exonuclease digestion was performed utilizing the Erase-a-base kit (Promega). Aliquots starting at the SacI site, whereas the deletions of the 3-kb insert were removed every 20 s. Samples of interest were transformed into DH10b. Clones with DNA of the appropriate size were identified by their migration on 0.7% agarose gels. Sequencing templates were prepared from 3 ml of culture using alkaline lysis (Sambrook et al., 1989) followed by adsorption and elution from an anion exchange column (Qiagen). Automated sequencing (Smith et al., 1986) was performed based on deoxynucleoside triphosphate chain termination (Sanger et al., 1977) using fluorescence-labeled M13 reverse primers (ABI) and an ABI model 370A interfaced with a Hewlett-Packard Vectra computer. Manual sequencing was also performed. The 1.5-kb PvuI-C fragment was subcloned into pUC19. Template was prepared by modification of the alkaline lysis procedure (Kraft et al., 1988). Four primers were utilized for sequencing in this region, 5' -CCATTGTTGACTAGATTG-3', 5' -CAAAGTCTCTACAGATG-3', 5' -GGGTTTCTCTTTAATGTCG-3', and 5' -GACTGTTGTATGACTGTGCC-3'. Double-stranded sequencing was performed using the Taq Trak Sequencing Kit (Promega). Sequencing reactions were run on 6% polyacrylamide gels containing 7 M urea (Sambrook et al., 1989). The fragments were transferred to Nytran (Schleicher and Schuell) following the manufacturer's instructions. Prehybridization and hybridization were carried out as described previously (Sambook et al., 1989). The probe was the yeast integrat-1990) from URAB diploids, digested to completion with HindIII, and serum were incubated at 23 °C for 1 h. The conditions for growth on glycerol at 37 °C was tightly linked to presence of the plasmid. Eighteen of these clones were mapped with several restriction enzymes and all clones were found to overlap in a region of 7 kb (Fig. 1). We found that this 7-kb region provided full complementation of both the growth defect and the vacuole segregation defect when introduced into vac1-1 on a single-copy plasmid. To demonstrate that this complementing fragment originated from the VAC1 locus and did not encode another yeast gene which could suppress the vac1-1 phenotype, the 2-kb BstEII fragment from within the 7-kb complementing region was subcloned into Ylp5, digested with KpnI (leaving approximately 1 kb on either side) and integrated into LWY147, a strain which is wild-type for VAC1 and contains the uracil auxotrophy ura3-52. Introduction of URA4 into the chromosome at the VAC1 locus was demonstrated by Southern analysis (data not shown). When the diploid formed from this modified strain and LWY148 was sporulated and tetrads dissected, it was found that the URA4 marker segregated away from the vac1-1 vacuole segregation defect in each of the 26 tetrads analyzed. This demonstrates that the original chromosomal location of the isolated VAC1 clone is tightly linked with the vac1-1 gene.

**Complementation Analysis**—To identify the location of the VAC1 gene within the original 7-kb region, subclones were tested for complementing activity. Two overlapping frag-
A set of nested deletions from each direction was obtained, and sequencing of the relevant clones was performed. The 1545 bases encode a protein of 515 amino acids. This amino-terminal third of the protein is not required for VACl protein function. Alternatively, the second methionine may be the initiating methionine. However, there is no TATA box located between the first and second ATG. It is also possible that two proteins are produced from a single transcript. Note that a construct with only five bases upstream of the AUG of the second methionine yielded full complementation of the vacu-1 vacuole segregation defect. This suggests that sufficient transcription is occurring from the neighboring E. coli sequences (perhaps the T3 promoter or the β-galactosidase promoter). These findings suggest that a very low level of transcription is needed for full complementation of vacu-1, i.e. that the VACl protein is only required in low copy number.

Sequence of the VACl Gene—The sequence of the VACl gene and the derived protein sequence is presented in Fig. 2. The 1545 bases encode a protein of 515 amino acids. This extended open reading frame is the only one among the six possible reading frames. Two possible TATA boxes (Oliver and Warmington, 1989), TATAAAT and TATATAT, are located 205 and 256 base pairs upstream of the first ATG. We found that the first 108 amino acids are not required for complementation of the VACl protein but is the type first identified in TFIIIA (Miller et al., 1985). The second two, starting X2-Cys-X4-Cys, and are similar to the sequence first identified in adenovirus ElA, which is X2-Cys-X4-Cys-X4-Cys-X4-Cys.

The FASTA and TFASTA programs of Pearson and Lipman (1988) were used to compare the derived VACl amino acid sequence of 515 residues with sequences in GenBank, Swiss EMBL, and the NBRF protein libraries. In addition, the DNA sequence was compared with sequences in GenBank and NBRF DNA libraries. No significant homologies with any known sequences were observed. The protein has no long stretches of hydrophobic amino acids, suggesting that it is not an integral membrane protein. In addition, there is no NH2-terminal leader sequence. Vactp has three zinc fingers. The first, starting at amino acid eight, has the pattern Cys-X2-Cys-X2-Cys-Phe-X2-Leu-X2-His-X2-His and is the type first identified in TFIIIA (Miller et al., 1985). The second two, starting at Cys-78 and Cys-221, have the pattern Cys-X2-Cys-X2-Cys-X2-Cys-Cys, and are similar to the sequence first identified in adenovirus ElA, which is Cys-X2-Cys-X2-Cys-X2-Cys. Interestingly, another gene involved in normal vacuole biogenesis, VPS18, has recently been identified which contains a single zinc finger Cys-X2-Cys-X2-Cys-X2-Cys-X2-Cys. VPS18 shows 70% identity in this region (Robinson et al., 1991) with yet a third gene involved in vacuole biogenesis, END1/PEPS (Dulic and Riezman, 1989; Woolford et al., 1990).
were digested with ClaI site were deleted and replaced with an 866-base construction of this strain, termed uacl-A2, was confirmed by a yeast strain in which the entire VAC1 coding region has which was hybridized with the 1.5-kb PstI fragment containing one-third of the VAC1 open reading frame plus flanking sequences (Fig. 4, lane 2). In the wild-type control, only the 2.1-kb fragment of wild-type VAC1 (lane 2), and an additional band at 4.7 kb.

Deletion of the Entire VAC1 Coding Region—To construct a yeast strain in which the entire VAC1 coding region has been deleted, 1617 base pairs between the BstEII site and the closer BclI site were deleted and replaced with an 866-base pair fragment containing the TRP1 marker (Fig. 1B). The construction of this strain, termed vac1-Δ2, was confirmed by Southern analysis. Genomic DNA from both the vac1-Δ2/VAC1 heterozygous diploid and a VAC1/VAC1 control were digested with excess ClaI and electrophoresed through a 0.7% agarose gel. The fragments were transferred to a membrane which was hybridized with the 1.5-kb PstI fragment containing one-third of the VAC1 open reading frame plus flanking sequence (Fig. 1A). As expected, the heterozygous diploid showed two bands (Fig. 4, lane 1), the wild-type fragment at 2.1 kb (Fig. 1A) and an additional band greater than 4 kb (Fig. 1B). In the wild-type control, only the 2.1-kb fragment is present (Fig. 4, lane 2). In Fig. 4, lane 1, the 2.1-kb fragment is much darker than the 4.7-kb fragment, because the latter has significantly less homology with the 1.5-kb PstI fragment used as the hybridization probe.

Deleting the entire VAC1 gene results in a viable yeast strain which is defective in vacuole segregation (Fig. 5). The strain is viable on YEPD at both 23 and 37 °C, although the doubling time in liquid culture at 23 °C is one-half that observed for wild-type cells and growth is very poor at 37 °C. Only microcolonies can form when this strain is grown on YEP glycerol plates at 37 °C. A second strain, vac1-Δ1, in which two-thirds of the coding region was removed, leaving only amino acids 1–152, was also constructed (Fig. 1C). The vacuole segregation defect in each of these strains were identical to each other and similar to, although more extreme than, that reported previously for vac1-1 (Fig. 4 of Weisman et al., 1990). Whereas the mother cells contain vacuoles that are approximately the same size as wild-type vacuoles, the bud vacuoles are either much smaller than normal or not detectable with either FITC or dichlorocarboxyfluorescein diacetate. Another phenotype which distinguishes the wild-type cells from the vac1-Δ2 mutant is that the vacuoles of wild-type mother cells are often irregularly shaped; two examples of wild-type cells with segregation structures can be seen (Fig. 5A). In the mutant, the vacuole in the mother cell is rounded, and no segregation structures can be seen (Fig. 5B). This difference in vacuole morphology and number of segregation structures between wild-type and mutant cells has also been reported for ups3 (Raymond et al., 1990).

Disruption of VPS3 has been reported to produce a yeast strain with the same phenotype as vac1-1 (Raymond et al., 1990). We find that the size and distribution of vacuoles in the bud was identical in ups3-Δ1 and vac1-Δ2 and that introduction of VAC1 on a multicopy plasmid does not suppress the ups3-Δ1 vacuole segregation defect (data not shown). Alleles of ups34, which have also been reported to cause a defect in vacuole segregation (Herman and Emr, 1990), are also not complemented by overexpression of VAC1. Vacuole inheritance in vac1-Δ1, as measured with the ade2 fluorophore in vegetatively growing cells and transfer of the ade2 fluorophore in yeast zygotes, was nearly identical to that reported for the original vac1-1 mutant strain (Weisman et al., 1990). Thus, although deleting the VAC1 gene has a profound effect
on vacuole segregation, the bud vacuoles eventually grow to a normal size sometime after cytokinesis and before bud emergence.

**Segregation of the Golgi Apparatus Is Normal in vac1-Δ1**—Since most mutants which are unable to sort proteins from the Golgi to the vacuole are still normal for vacuole segregation into the bud, why is a small class of mutants defective in both processes? If the primary defect were in Golgi segregation and if the presence of a Golgi is required for each aspect of normal vacuole maintenance, then the conjunction of defects in vacuole segregation, vacuole protein sorting, and Golgi segregation should be observed. This is not the case for vac1-Δ1. Since Golgi segregation appears to be normal in vac1-Δ1 (Fig. 6) and is also normal in ups3 (data not shown).

**DISCUSSION**

The sequence of the VAC1 protein has neither the apolar domains characteristic of integral membrane proteins nor a NH₂-terminal leader sequence, characteristic of proteins which enter the secretory pathway. However, two lines of evidence suggest that Vac1p is not freely diffusible in the cytosol. In zygotes, the transfer of ade2 fluorophore from a parental vacuole into the bud, founding the new bud vacuole, is defective when both parents of the zygote are vac1-1 (Weisman and Wickner, 1988). This transfer normally occurs after bud emergence, well after nuclear fusion and cytoplasmic mixing have occurred. However, when one of the parents of the zygote is vac1-1 and the other is VAC1, the vacuole from the vac1-1 parent cannot participate in intervacuole tubulovesicular traffic, whereas the vacuole derived from the VAC1 parent shows no defect. This observation suggests that the Vac1p is bound to cellular structures such as membranes or cytoskeleton and not freely diffusible.

A second independent line of evidence comes from recent studies of an in vitro reaction which reflects processes of vacuole inheritance. In this reaction, the vacuoles of semi-intact cells produce striking extensions with the appearance of long tubules or strings of vesicles. This reaction requires the VAC1 and VAC2 proteins and incubation at 25–37 °C. When both the semi-intact cells and cytosol are prepared from vac1-1 or vac1-Δ2 strains, no reaction is seen at 37 °C. However, vac1-1 cytosol supports the formation of tubulovesicular structures by vacuoles from wild-type semi-intact cells, whereas wild-type cytosol complements vac1-1 semi-intact cells. These data support the inference from the in vivo studies, cited above, that the VAC1 protein is partially bound to cytoskeleton or membrane. We hypothesize, as a first postulate of Vac1p function, that it is part of the complex which tethers the vacuole membrane to microtubules, either directly or via kinetinor dynemin-like motor proteins. This postulate may be tested both by immunofluorescence, using the VAC1 sequence as a guide to the generation of antibody, and by fractionation and reconstitution of the in vitro reaction of formation of tubulovesicular structures.

The VAC1 gene encodes a protein of 515 amino acyl residues which is without strong homolog in the three major sequence data banks. The novelty of the VAC1 protein sequence is not surprising, as to date few genes involved in organelle segregation have been isolated. The only clue to function from the sequence is the fact that Vac1p contains three zinc fingers. The first zinc finger is almost identical with the zinc finger motif first identified in Xenopus laevis TFIIIA. Not only does this VAC1 zinc finger have the conserved cysteines and histidines with the correct spacing, but it also has the conserved phenylalanine and leucine which have been proposed to be the hallmarks of true transcription factors (Evans and Hollenberg, 1988). The only conserved amino acid of this zinc finger motif which is missing from VAC1 is a phenylalanine or tyrosine two amino acids before the first cysteine. In Vac1p this residue is a valine. The major difference between Vac1p and the known transcription factors is that this zinc finger occurs only once in VAC1, yet occurs two to nine times in the transcription factors (Evans and Hollenberg, 1988). The other two zinc fingers in Vac1p are very similar to the type first reported for adenovirus E1A. The major difference in this case is that E1A has 13 amino acids between the two sets of cysteines, whereas in VAC1 there are 12 amino acids. Although E1A does not appear to bind to a specific site on DNA (Berk, 1986), it is intimately involved in transcription and this cysteine-rich region is clearly important for its function (Lilie et al., 1987). Likewise, most other proteins in which this motif has been identified are DNA associated proteins (Frankel and Pabo, 1988).

These observations raise a second possibility of Vac1p function, that it is a nuclear transcription factor. Our enthusiasm for this postulate is tempered by the fact that, although the sequence of at least nine genes involved in vacuole biogenesis is known (Rothman et al., 1990; Herman et al., 1991; Raymond et al., 1990; Duic and Riezman, 1989; Woolford et al., 1990; Wada et al., 1990; Banta et al., 1990; Herman and Emr, 1990; Robinson et al., 1991; this report), three of these genes have been found to contain zinc fingers. It seems unlikely that all are involved in vacuole biogenesis-related transcription. Rather, zinc may be involved in either the structure of Vac1p, its interactions with other proteins, or its catalytic function. As more types of proteins with this motif are discovered, the various roles of zinc fingers may emerge.

Aside from their effect on vacuole segregation, the vac1 mutants secrete carboxypeptidase Y instead of localizing it to the vacuole, as initially required by the selection (Weisman et al., 1996). Recently, two other vps mutants with a vac phenotype have also been reported, vps34 (Raymond et al., 1990) and vps34 (Herman and Emr, 1990). What these three mutants have in common is a defect in vacuole segregation as well as localization of carboxypeptidase Y to the cell surface instead of the vacuole. Each mutant grows, albeit much more slowly, in the absence of their respective wild-type gene products. Interestingly, all three mutants have only a partial block in vacuole segregation. As was reported with vac1-1, these cells acquire a normal size vacuole before producing a bud despite the fact that they inherited little or no parental vacuolar material. Vps3p and Vps34p are partially associated with membranes or cytoskeleton, as judged by differential centrifugation of lysates (Raymond et al., 1990; Herman and

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2 B. Conradt, J. Shaw, T. Vida, S. D. Emr, and W. Wickner, manuscript in preparation.
Emr, 1990). Although upe3 and upe34 mutants have a similar phenotype, no genetic interactions between these mutants have been observed. Our studies of the behavior of Vac1p in vitro also suggest that it is probably a peripheral membrane protein or associated with cytoskeleton. The lack of an obvious membrane spanning domain or leader sequence in Vac1p is consistent with such a localization.

In this paper we report that vac1-Δ2 is wild-type with regards to Golgi segregation. This observation extends our initial findings that both mitochondrial segregation and nuclear division are normal in vac1. There is reason to believe that mutants could be isolated that are defective in both Golgi and vacuole segregation, since vacuole segregation and Golgi segregation occur at a similar point in the cell cycle (Redding et al., 1991; Weisman et al., 1987). It is possible that Vac1p is responsible for sorting a protein directly involved in vacuole segregation to the cytoplasmic face of the vacuole membrane. Alternatively, Vac1p may be independently involved in Golgi to vacuole protein sorting and vacuole segregation. We favor this second hypothesis. First, of approximately 50 mutants defective in carboxypeptidase Y localization to the vacuole, only two have been reported to be defective in vacuole segregation (Raymond et al., 1990, Herman and Emr, 1990). If mistargeting generally leads to a segregation defect, then this phenotype should have been more common. Furthermore, in vitro studies show that wild-type cytosol (but not vac1-1 cytosol) can complement the ability of vac1-1 vacuoles to form tubulovesicular structures. These data are most simply explained by the Vac1p being a physically bound element of the cytoskeleton or vacuole. More direct studies, employing antibody and entailing purification of functional Vac1p, will be needed to test this postulate.

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