New Components of a System for Phosphate Accumulation and Polyphosphate Metabolism in *Saccharomyces cerevisiae* Revealed by Genomic Expression Analysis

Nobuo Ogawa,* Joseph DeRisi,† and Patrick O. Brown*‡

*Department of Biochemistry, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California 94305-5307; and †Department of Biochemistry and Biophysics, University of California at San Francisco, 513 Parnassus Ave., San Francisco, California 94143

The PHO regulatory pathway is involved in the acquisition of phosphate (P_i) in the yeast *Saccharomyces cerevisiae*. When extracellular P_i concentrations are low, several genes are transcriptionally induced by this pathway, which includes the Pho4 transcriptional activator, the Pho80-Pho85 cyclin-CDK pair, and the Pho81 CDK inhibitor. In an attempt to identify all the components regulated by this system, a whole-genome DNA microarray analysis was employed, and 22 PHO-regulated genes were identified. The promoter regions of 21 of these genes contained at least one copy of a sequence that matched the Pho4 recognition site. Eight of these genes, *PHM1–PHM8*, had no previously defined function in phosphate metabolism. The amino acid sequences of *PHM1 (YFL004w)*, *PHM2 (YPL019c)*, *PHM3 (YJL012c)*, and *PHM4 (YER072w)* are 32–56% identical. The *phm3* and *phm4* single mutants and the *phm1 phm2* double mutant were each severely deficient in accumulation of inorganic polyphosphate (polyP) and P_i. The phenotype of the *phm5* mutant suggests that *PHM5 (YDR452w)* is essential for normal catabolism of polyP in the yeast vacuole. Taken together, the results reveal important new features of a genetic system that plays a critical role in P_i acquisition and polyP metabolism in yeast.

INTRODUCTION

Phosphate (P_i) is an essential nutrient for all organisms, used in the biosynthesis of diverse cellular components, including nucleic acids, proteins, lipids, and sugars. It is therefore essential for organisms to have evolved regulatory mechanisms for acquisition, storage, and release of this molecule (Torriani-Gorini *et al.*, 1994).

In *Saccharomyces cerevisiae,* the PHO regulatory pathway regulates expression of the "PHO" genes, involved in the scavenging and specific uptake of P_i from extracellular sources (Johnston and Carlson, 1992; Oshima, 1997). The PHO regulatory system consists of at least five PHO-specific regulatory proteins, the Pho2 and Pho4 transcriptional activators, the Pho80-Pho85 cyclin-cyclin dependent protein kinase (CDK) complex, and the Pho81 CDK inhibitor (Figure 1). Pho84 is a high-affinity P_i transporter localized on plasma membrane, which has been shown to contribute to P_i uptake from culture medium (Bun-ya *et al.*, 1991). *PHO84* gene

expression is activated by a P_i-starvation signal mediated by the PHO regulatory system. Additionally, the *PHO5* gene encodes a repressible acid phosphatase which is localized to the periplasmic space.

When the P_i concentration in the medium is low ($\sim$ 0.2 mM P_i), the Pho81 protein inhibits the Pho80-Pho85 kinase activity, which in its active state catalyzes a hyperphosphorylation of Pho4 (Schneider *et al.*, 1994; Ogawa *et al.*, 1995). The hypophosphorylated form of Pho4 is preferentially localized to the nucleus, where together with Pho2, it activates target gene transcription (Kaffman *et al.*, 1998; Komeili and O'Shea, 1999). Alternatively, when the P_i concentrations are high ($\sim$ 10 mM P_i), the Pho80-Pho85 kinase phosphorylates Pho4. In addition to having a lower affinity for Pho2 and the nuclear import protein Pse1/Kap121, phosphorylated Pho4 is a preferred substrate of the nuclear export protein Msn5, resulting in extranuclear localization. Phosphorylated Pho4 is thus unable to activate target gene expression.

Besides *PHO5* and *PHO84*, seven additional genes are known to be regulated by the PHO regulatory system; these include *PHO11* and *PHO12* (homologs of *PHO5*), *PHO8* (vacuolar alkaline phosphatase, Kaneko *et al.*, 1987), *PHO89* (Na/P_i cotransporter, Martinez and Persson, 1998), *PHO86*

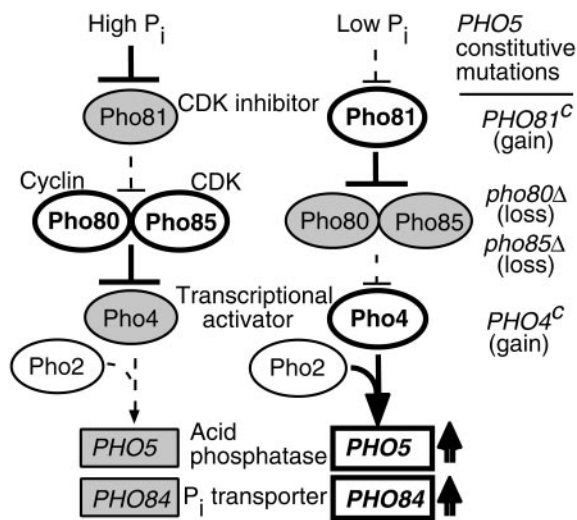‡ Corresponding author: E-mail address: pbrown@cmgm.stanford.edu.

Figure 1. PHO regulation system. A simplified schematic of the PHO regulation mechanism showing the five main regulator proteins is illustrated (Johnston and Carlson, 1992; Oshima, 1997). Ovals and boxes represent proteins and genes, respectively. Thick lines mean that the signals are transduced to the downstream component, while dotted lines indicate the absent of an interaction with the downstream component. Open ovals and boxes indicate active states, gray oval and boxes indicate inactive state. The PHO5-constitutive mutants used in this study are listed to the right of the corresponding mutated protein with the nature of the mutation indicated by "gain" or "loss" of function.

(required for P_i uptake, Yompakdee *et al.*, 1996), *PHO81* and *SPL2* (a homolog of *PHO81*, Flick and Thorner, 1998). The promoters of all nine previously recognized PHO-regulated genes have common motifs, CACGTG and/or CACGTT, as core sequences comprising the Pho4 binding site (Oshima, 1997). Both the regulating properties and the functions of the target genes point to the critical role played by the PHO regulatory system in P_i acquisition in yeast. Comprehensive identification and characterization of the PHO-regulated genes in the yeast genome is therefore likely to be an important step toward understanding the regulation and physiology of P_i metabolism.

DNA microarrays provide a systematic way to study the expression programs of the entire genome (DeRisi *et al.*, 1997). Using DNA microarrays, we conducted an exhaustive search for yeast genes regulated by the PHO regulatory system. Several of the genes identified were of unknown function and were further characterized by gene disruption. Biochemical analysis of five of the novel PHO-regulated genes revealed them to be important in inorganic polyphosphate (polyP) metabolism.

PolyP, a linear polymer of up to hundreds of P_i residues linked by high-energy phosphoanhydride bonds, is ubiquitous in nature, having been found in all organisms examined (Kornberg, 1999). *S. cerevisiae* is known to accumulate large amounts of polyP in vacuoles, comprising 37% of the total cellular phosphate (Urech, 1978). The enzyme primarily responsible for polyP synthesis in *Escherichia coli* is polyP kinase (PPK), and polyP is hydrolyzed to P_i by exopolyphosphatase (Kornberg, 1999). In *S. cerevisiae*, an exopolyphos-

phatase gene, *PPX1*, has been identified (Wurst *et al.*, 1995), but a gene for PPK has not. Moreover, no gene homologous to the bacterial PPKs has been found in the genome databases of *S. cerevisiae*. Thus, the identify of the enzymes that mediate polyP metabolism in yeast and the other eukaryotes has been an important unsolved problem in metabolism of a centrally important nutrient.

MATERIALS AND METHODS

Strains and Media

S. cerevisiae strains used in this study are listed in Table 1. YPAD medium (Adams *et al.*, 1997) was used as rich media. A P_i-depleted YPAD medium (YPAD-Pi) was prepared as described (Kaneko *et al.*, 1982), and a high-P_i YPAD (YPAD+Pi) medium was prepared by addition of sodium phosphate (10 mM, pH 5.8) to the YPAD-Pi medium. High-P_i, low-P_i, and P_i-free synthetic media were prepared as described (Yoshida *et al.*, 1989). YPAD medium buffered at pH 7.5 supplemented with 50 mM $CaCl_2$ was prepared as described (Zhang *et al.*, 1998).

Growth Conditions for RNA Preparation

For comparison of the gene expression pattern between the low- and high-P_i conditions, yeast strain NBW7 or DBY7286 was grown in 25 ml of the YPAD+Pi medium overnight, the cells were collected, washed with the YPAD-Pi medium two times, and inoculated into 500 ml of the YPAD+Pi and -Pi media to an OD_{600} of 0.05. The two cultures were shaken at 30°C to an OD_{600} of 0.5, the grown cells were harvested, and then frozen by immersion in liquid nitrogen. The cells were stored in −80°C until RNA was prepared. For comparison of the gene expression pattern between the mutants and wild-type, yeast strains NBD82–1 ($PHO4^c$-1), NBD80–1 ($pho80\Delta$), NBD85A-1 ($pho85\Delta$), NOF1 ($PHO81^c$-1), and NBW7 (wild-type) were precultivated in 25 ml of YPAD overnight, the grown cells were collected washed with YPAD, and inoculated into 500 ml of YPAD to an OD_{600} of 0.1. The remainder of the procedure was the same as above.

DNA Microarray Analysis

The preparation of the yeast ORF DNA microarray, RNA preparation from yeast cells, probe preparation with fluorescence dye, hybridization, scanning of the hybridized array, and data processing were performed as described previously (Chu *et al.*, 1998; Spellman *et al.*, 1998). To compare gene expression patterns between the low- and high-P_i conditions, Cy3- or Cy5-labeled cDNA probes were prepared from the high- or low-P_i samples, respectively, by reverse transcription in the presence of Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech., Piscataway, NJ), as previously described (DeRisi *et al.*, 1997). To compare the gene expression patterns between mutant and wild-type cells, cDNA probes templated by mRNAs prepared from wild-type and mutant cells were labeled with Cy3-dUTP and Cy5-dUTP, respectively. The original array data are available on our web page on <http://cmgm.stanford.edu/pbrown/phosphate/>.

Construction of the S. cerevisiae Disruption Mutants

To make disruption mutants of *PHM1*, *PHM2*, *PHM3*, *PHM4*, *PHM5*, *PHM6*, and *CTF19*, PCR-mediated gene disruptions were performed as described (Sakumoto *et al.*, 1999). pCgHIS3, pCgTRP1, pCgLEU2 harboring the *Candida glabrata HIS3*, *TRP1*, and *LEU2* fragments on pUC19, respectively, were obtained from Dr. Y. Mukai (Osaka University), and used as templates to generate the PCR fragments for the gene disruptions. Deletion regions in the target

Table 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Reference
NBW7	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1*	Ogawa and Oshima, (1990)
NBD4-1[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 pho4Δ::HIS3*	Ogawa and Oshima, (1990)
NBD82-1[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 PHO4^c-1*	Ogawa and Oshima, (1990)
NBD80-1[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 pho80Δ::HIS3*	Bun-ya *et al.*, (1991)
NBD85A-1[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 pho85Δ::ADE2*	Our stock
NOF1[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 PHO81^c-1*	Our stock
NBD8184-1B[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 pho84Δ::HIS3*	Our stock
NBM4Lf1[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 phm1Δ::CgLEU2*	This study
NBM19Hf1[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 phm2Δ::CgHIS3*	This study
NBM4L19H2[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 phm1Δ::CgLEU2 phm2Δ::CgHIS3*	This study
NBM12W2[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 phm3Δ::CgTRP1*	This study
NBM72W7[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 phm4Δ::CgTRP1*	This study
NBM452H1[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 phm5Δ::CgHIS3*	This study
NBM281H1[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 phm6Δ::CgHIS3*	This study
NBM18H7[a]	*MAT*a *ade2 his3 leu2 trp1 ura3 pho3-1 ctf19Δ::CgHIS3*	This study
DBY7286	*MAT*a *ura3-52*	Our stock
CRY	*MAT*a *ade2 his3 leu2 trp1 ura3*	Wurst *et al.*, (1995)
CRX[b]	*MAT*a *ade2 his3 leu2 trp1 ura3 ppx1Δ::LEU2*	Wurst *et al.*, (1995)
CB023[b]	*MAT*a *ade2 his3 leu2 trp1 ura3 pep4Δ::HIS3 prb1Δ::hisG prc1Δ::hisG*	Wurst *et al.*, (1995)
CRY-V4[b]	*MAT*a *ade2 his3 leu2 trp1 ura3 vma4Δ::URA3*	Wurst *et al.*, (1995)
NB91-6B	*MAT*a *his3 leu2 trp1 pep4 (=pho9-1)*	Our stock

[a] These strains were derived from strain NBW7.
[b] These strains were derived from strain CRY.

genes were as follows: *PHM1*, +1 to +2387; *PHM2*, +1 to +2508; *PHM3*, +53 to +926; *PHM4*, +44 to +347; *PHM5*, +1 to +2025; *CTF19*, +1 to +1130; *PHM6*, +1 to +315 (nucleotide positions relative to A of initiation codon of ATG in each ORF). In each case, the deleted sequence was replaced with the marker genes indicated in Table 1. The disruption junctions were verified by colony-PCR (Adams *et al.*, 1997).

Polyphosphate Overplus

Polyphosphate overplus (Harold, 1966) culture was performed as follows: The yeast strains indicated were grown in the YPAD-Pi media overnight, the grown cells were collected, washed with water, and resuspended in 10 ml of the synthetic P$_i$-free media to an OD$_{600}$ of 0.5. The cultures were shaken for 2 h. at 30°C, then potassium phosphate (pH 5.8) was added (10 mM final concentration). After 2 more hours of cultivation, the cells were harvested, and then frozen by immersion in liquid nitrogen. For the polyP overplus at low pH, yeast strains were grown in the YPAD-Pi media overnight, the grown cells were collected, washed with water, and resuspended in 10 ml of the YPAD media supplemented with 10 mM (final) KH$_2$PO$_4$ and/or 10 mM (final) sodium acetate buffer (pH 4.0) to an OD$_{600}$ of 0.5. The cultures were shaken for 2 h at 30°C, then the cells were harvested, and frozen by immersion in liquid nitrogen.

Cell Extract Preparation From Yeast

Cells in 150 μl of extraction buffer (50 mM Tris-HCl [pH 7.4], 100 mM KCl, 1 mM EDTA) were mixed in a vortex mixer with 150 mg of acid-washed glass beads (0.5-mm diameter) for 2 min at 4°C, and microcentrifuged at 14,000 rpm for 10 min at 4°C. The aqueous phase was extracted by phenol:chloroform followed by chloroform and ether extractions. After removing ether in the samples by evaporation, their A$_{260}$ values were measured to calculate their total RNA concentrations.

PolyP Detection by PAGE

PolyP analysis by PAGE was performed as described in Wurst *et al.* (1995) with the following modifications. Samples containing the indicated amounts of RNA were loaded with 7 μl of loading dye solution (1x TBE buffer [Sambrook *et al.*, 1989], 10% sucrose, 0.05% bromophenol blue) on a 20% polyacrylamide gel (270-mm height x 165-mm wide x 0.5-mm thick) with 1x TBE buffer. Ten μg of sodium phosphate glass type 5, 15, or 35 (Sigma, St. Louis, MO) were loaded as a polyP size markers. Radioactive [γ-^{32}P]ATP was also used as an additional size marker. The electrophoresis was run at 20 V/cm for ~ 1.5 or 3 h, and the ATP-loaded gel piece was analyzed by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The nonradioactive piece of gel was soaked into 10% acetate, 10% methanol for 15 min, stained with the staining solution (0.5% Toluidine Blue O [Sigma], 25% methanol, 5% glycerol, 5% acetate) for 15 min, and destained with destaining solution (same as the staining without Toludine Blue O) for 10 min several times. ATP migrates at a position corresponding to polyP of between seven and eight P$_i$ residues long. PolyP bands of sodium phosphate glass type P35 with 55 and 65 chain length on PAGE were extracted and used as markers to estimate longer chain lengths of polyP.

Enzymatic polyP Assays

PolyP was assayed by the nonradioactive method as described (Ault-Riché *et al.*, 1998), without the Glassmilk purification steps. Since the major population of yeast polyP is < 60 P$_i$ residues in length (Figure 4B), they are not able to be effectively trapped by Glassmilk. Concentrations of polyP in the samples were shown as mol of P$_i$ residues per mg of total RNA.

PHM2-GFP Fusion Experiment

pPHM2-GFP was constructed by insertion of the PCR fragment corresponding to nucleotide positions from − 393 to + 2505 of *PHM2* (relative to A of initiation codon of ATG) into an *Eco*RI-

*Hin*dIII gap of pTS395, a *GFP*-expressing yeast vector provided by Dr. D. Botstein. Yeast transformant NBM-4L19H1[pPHM2-GFP] was grown in synthetic low-P_i medium lacking uracil for > 3 h at 30°C. Fluorescent dye, FM4–64 (Molecular Probes, Eugene, OR) , which has been reported to stain vacuolar membrane (Vida and Emr, 1995), was added to 30 μM, and incubated for an additional 15 min. Ten-microliter samples were examined by fluorescent microscope Axioplan2 (Carl Zeiss, Thomwood, NY) following the procedure of Vida and Emr (1995) and Adams *et al.* (1997).

P_i Uptake Assay

A P_i uptake assay was performed following the procedure of Bun-ya *et al.* (1991). Yeast strains were grown in the YPAD-P_i media overnight, the grown cells were collected, washed with water, and resuspended in 50 ml of synthetic P_i-free media to an OD_{660} of 0.1. The cultures were shaken for 2 h at 30°C, and their OD_{660} values were remeasured. Potassium phosphate (0.1 mM, pH 5.8) and radioactive phosphate ($^{32}PO_4$; 1 μCi/ml) were added to cultures, and shaken. Five-milliliter samples were taken at indicated intervals and immediately filtered though a HA filter (3.5-mm diameter, Millipore). The cells trapped on the filter were washed with 10 ml of synthetic P_i-free media. The radioactivity trapped on the membrane filters was quantitated by a liquid scintillation counting.

RESULTS

Whole Genome Survey for the PHO-Regulated Genes

In order to identify all of the genes regulated by the PHO regulatory pathway, we used DNA microarrays fabricated as described previously (DeRisi *et al.*, 1997, Chu *et al.*, 1998; DeRisi *et al.*, 1997, Chu *et al.*, 1998; Spellman *et al.*, 1998). A cDNA probe prepared from poly(A)$^+$ RNA isolated from a wild-type yeast strain, NBW7, cultivated in low-P_i media (YPAD-Pi), was labeled with Cy5 fluorescent dye, while a cDNA probe prepared from the same strain cultivated in high-P_i media (YPAD+Pi) was labeled with Cy3 fluorescent dye. The Cy5- and Cy3-labeled cDNA probes were mixed and hybridized to the microarray. As expected, the transcript levels of both *PHO5* and *PHO84* were elevated in low-P_i media in two independent experiments (Figure 2A). In a repeat experiment using strain DBY7286, derived from S288C, (the standard *S. cerevisiae* strain for the genome database), most of the highly derepressed genes we found in NBW7 were consistent with those identified in DBY7286 (Figure 2) suggesting similar PHO regulation in the two strains.

To evaluate if these gene expression changes were dependent on the known PHO regulatory factors, further DNA microarray analyses were performed comparing the parental strain NBW7 to four strains carrying mutations in components of the PHO regulatory system (Figure 1): 1) a gain-of-function mutation of the *PHO4* gene encoding transcriptional activator named *PHO4^c-1* (Ogawa and Oshima, 1990); 2) a *pho80* deletion mutant; 3) a *pho85* deletion mutant; and 4) *PHO81^c-1* a gain-of-function mutation in the *PHO81* gene, encoding the CDK inhibitor for Pho80-Pho85 (Ogawa *et al.*, 1995). In all cases *PHO5* was expressed (Figure 2A) irrespective of P_i concentration (YPAD media). A total of eight DNA microarray experiments were performed, including two independent repeated experiments. Cluster analysis of the combined data is shown in Figure 2B. Transcript

levels of > 80 genes changed significantly in response to one or more of the conditions we total.

All nine genes that have previously been reported to be PHO-regulated (*PHO5, PHO11, PHO12, PHO8, PHO84, PHO89, PHO86 PHO81* and *SPL2*) were successfully identified (Figure 2B). *PHO5, PHO11, PHO12, PHO84, PHO89,* and *SPL2* had high differential expression ratios (more than five-fold) whereas *PHO8* and *PHO86* had lower differential ratios (between two and fivefold), consistent with previous results from Northern analysis (Kaneko *et al.*, 1987; Bun-ya *et al.*, 1991; Yompakdee *et al.*, 1996). The nine known PHO-regulated genes can be categorized into three groups according to their function (Table 2). A third member of the *PHO81* family, *YPL110c*, also seems to be regulated by the PHO regulatory pathway (Figure 2B) in a manner similar to *PHO81*. Twelve additional genes showed clear evidence of PHO regulation in our experiments (Figure 2B and Table 2). Three of these genes (*YPL019c, YJL012c* and *YER072w*) had levels of differential expression similar to *PHO5* and *PHO84*, in nearly all of the eight experiments. Interestingly, the putative proteins encoded by these three genes share homology with *YFL004w* as described later (Figure 3A). The expression of *YFL004w* was also regulated by the PHO pathway, with differential expression levels similar to *PHO8* and *PHO86* (Figure 2B). Because of their homology and high differential expression ratios, we speculated that these four genes, which we have named *PHM1* through *PHM4* (*phos*phate *m*etabolism genes), might have important functions in P_i metabolism.

Among the other newly identified PHO-regulated genes listed in Table 2, the functions of three had been previously described: *HOR2/GPP2* (*YER062c*), *CTF19* (*YPL018w*), and *HIS1* (*YER055c*). The *HOR2/GPP2* gene encodes glycerol phosphatase (Norbeck *et al.*, 1996) which hydrolyzes the phosphate bond of glycerolphosphate, releasing free P_i. The His1 enzyme (ATP phosphoribosyltransferase)-catalyzed reaction can produce pyrophosphate from ATP and phosphoribosylpyrophosphate. While both *HIS1* and *HOR2* encode enzymes that catalyze reactions that release P_i, the special significance of these reactions for the cell's P_i economy is not obvious. The Ctf19 protein has previously been identified as a subunit of the centromere-binding complex (Ortiz *et al.*, 1999), and has no recognized role in P_i metabolism or regulation. A possible explanation for the observed differential expression could be that *CTF19* shares promoter sequences with the "head-to-head" oriented adjacent gene *YPL019c/PHM2*, which was highly differentially expressed under PHO regulation. More plausibly, it may have an additional, unrecognized role in P_i metabolism.

Two "core" Pho4 binding sequences, CACGTG and CACGTT have previously been described. At least one of these sequences is found within 500 bp upstream of the coding sequence of 21 of the 22 putative PHO-regulated genes listed in Table 2, a fraction significantly higher than the 20% of all yeast ORFs that have one of these sequences within 500 bp upstream (1287 ORFs out of 6282). Of the 14 genes with more than two putative Pho4 binding sites, 9 had the high derepression ratios described above. Higher levels of differential expression showed some correlation to the presence of both of the two types Pho4 binding sites. Of the 22 putative PHO-regulated genes, only *YER038c* had no consensus Pho4-binding sites in its promoter region. The 3′
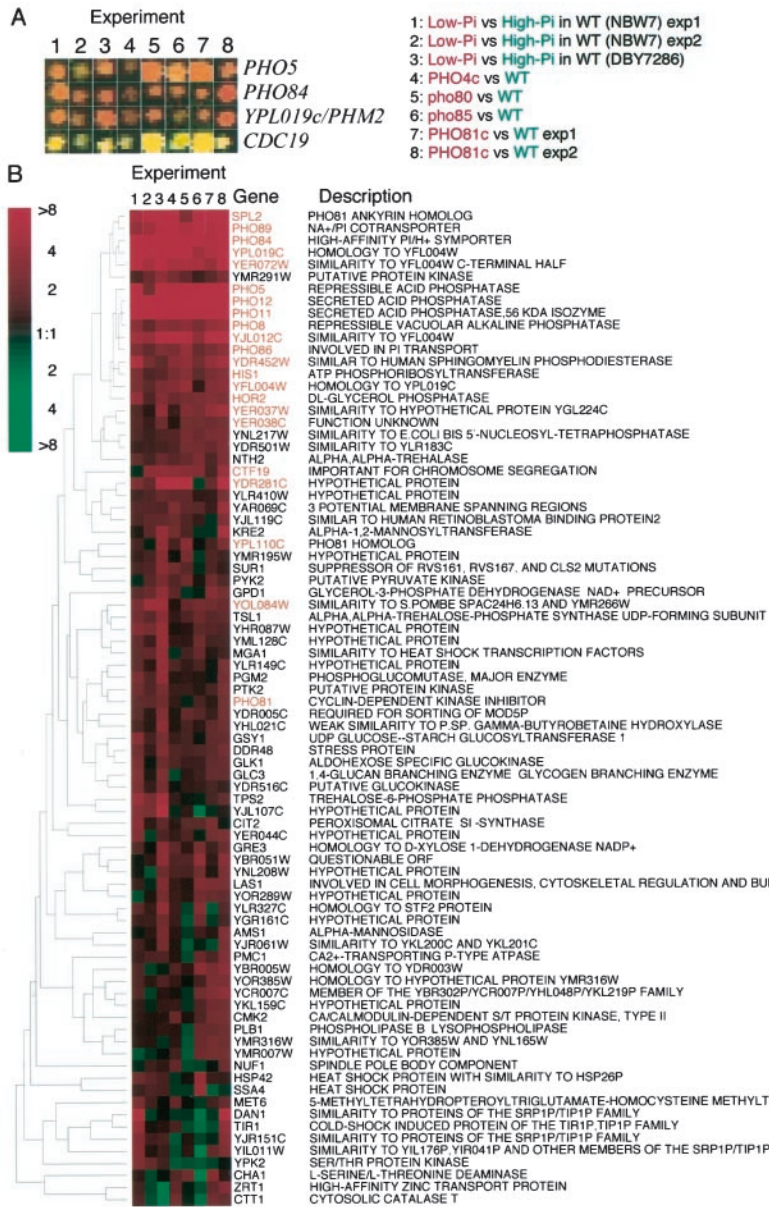
Figure 2. DNA microarray analysis. (A) Fluorescent scanning images of the spots for the typical PHO-regulated genes, *PHO5*, *PHO84*, and *YPL019c/PHM2*, and a non PHO-regulated gene, *CDC19*. The column number indicates experiments performed as follows, 1 and 2: comparison between NBW7 (wild-type) cultivated in low- (YPAD-Pi, Cy5) and high-P$_i$ media (YPAD+Pi, Cy3); 3: DBY7286 (wild-type) cultivated in low- (YPAD-Pi, Cy5) and high-P$_i$ media (YPAD+Pi, Cy3); 4: comparison between NBD82–1 (*PHO4^c-1*, Cy5) and NBW7 (Cy3); 5: comparison between NBD80–1 (*pho80Δ*, Cy5) and NBW7 (Cy3); 6: comparison between NBD85A-1 (*pho85Δ*, Cy5) and NBW7 (Cy3); 7 and 8: comparison between NOF1 (*PHO81^c-1*, Cy5) and NBW7 (Cy3). All strains used in experiment 4, 5, 6, 7, and 8 were cultivated in YPAD media. (B) Cluster analysis of the DNA microarray data. Eight independent DNA microarray data were analyzed the Cluster program (Eisen *et al.*, 1998). Genes showing more than twofold induction in any experiments correspond to the rows, and the experiments are the columns. Red represents a higher level of expression in the low-P$_i$ conditioned wild-type cells compared with the high-P$_i$ conditioned cells or in the mutant strains compared with the wild-type strains. The color saturation represents the magnitude of the expression ratio, as indicated by the scale at the top left of the figure. Black indicates no detectable difference in expression levels; gray denotes a missing observation. The rows, representing series of observation on individual genes, were ordered based on the similarity of their expression patterns in the eight experiments (Eisen *et al.*, 1998). The dendrogram to the left of the figure represents the correlation between expression patterns, as described by Eisen *et al.*, (1998). Names of the genes listed in Table 2 are emphasized by red color.

Figure 2.

A. Experiment 1 2 3 4 5 6 7 8 — *PHO5*, *PHO84*, *YPL019c/PHM2*, *CDC19*

1: Low-Pi vs High-Pi in WT (NBW7) exp1
2: Low-Pi vs High-Pi in WT (NBW7) exp2
3: Low-Pi vs High-Pi in WT (DBY7286)
4: PHO4c vs WT
5: pho80 vs WT
6: pho85 vs WT
7: PHO81c vs WT exp1
8: PHO81c vs WT exp2

B. Experiment 1 2 3 4 5 6 7 8

Gene	Description
SPL2	PHO81 ANKYRIN HOMOLOG
PHO89	NA+/PI COTRANSPORTER
PHO84	HIGH-AFFINITY PI/H+ SYMPORTER
YPL019C	HOMOLOGY TO YFL004W
YER072W	SIMILARITY TO YFL004W C-TERMINAL HALF
YMR291W	PUTATIVE PROTEIN KINASE
PHO5	REPRESSIBLE ACID PHOSPHATASE
PHO12	SECRETED ACID PHOSPHATASE
PHO11	SECRETED ACID PHOSPHATASE,56 KDA ISOZYME
PHO8	REPRESSIBLE VACUOLAR ALKALINE PHOSPHATASE
YJL012C	SIMILARITY TO YFL004W
PHO86	INVOLVED IN PI TRANSPORT
YDR452W	SIMILAR TO HUMAN SPHINGOMYELIN PHOSPHODIESTERASE
HIS1	ATP PHOSPHORIBOSYLTRANSFERASE
YFL004W	HOMOLOGY TO YPL019C
HOR2	DL-GLYCEROL PHOSPHATASE
YER037W	SIMILARITY TO HYPOTHETICAL PROTEIN YGL224C
YER038C	FUNCTION UNKNOWN
YNL217W	SIMILARITY TO E.COLI BIS 5'-NUCLEOSYL-TETRAPHOSPHATASE
YDR501W	SIMILARITY TO YLR183C
NTH2	ALPHA,ALPHA-TREHALASE
CTF19	IMPORTANT FOR CHROMOSOME SEGREGATION
YDR281C	HYPOTHETICAL PROTEIN
YLR410W	HYPOTHETICAL PROTEIN
YAR069C	3 POTENTIAL MEMBRANE SPANNING REGIONS
YJL119C	SIMILAR TO HUMAN RETINOBLASTOMA BINDING PROTEIN2
KRE2	ALPHA-1,2-MANNOSYLTRANSFERASE
YPL110C	PHO81 HOMOLOG
YMR195W	HYPOTHETICAL PROTEIN
SUR1	SUPPRESSOR OF RVS161, RVS167, AND CLS2 MUTATIONS
PYK2	PUTATIVE PYRUVATE KINASE
GPD1	GLYCEROL-3-PHOSPHATE DEHYDROGENASE NAD+ PRECURSOR
YOL084W	SIMILARITY TO S.POMBE SPAC24H6.13 AND YMR266W
TSL1	ALPHA,ALPHA-TREHALOSE-PHOSPHATE SYNTHASE UDP-FORMING SUBUNIT
YHR087W	HYPOTHETICAL PROTEIN
YML128C	HYPOTHETICAL PROTEIN
MGA1	SIMILARITY TO HEAT SHOCK TRANSCRIPTION FACTORS
YLR149C	HYPOTHETICAL PROTEIN
PGM2	PHOSPHOGLUCOMUTASE, MAJOR ENZYME
PTK2	PUTATIVE PROTEIN KINASE
PHO81	CYCLIN-DEPENDENT KINASE INHIBITOR
YDR005C	REQUIRED FOR SORTING OF MOD5P
YHL021C	WEAK SIMILARITY TO P.SP. GAMMA-BUTYROBETAINE HYDROXYLASE
GSY1	UDP GLUCOSE--STARCH GLUCOSYLTRANSFERASE 1
DDR48	STRESS PROTEIN
GLK1	ALDOHEXOSE SPECIFIC GLUCOKINASE
GLC3	1,4-GLUCAN BRANCHING ENZYME GLYCOGEN BRANCHING ENZYME
YDR516C	PUTATIVE GLUCOKINASE
TPS2	TREHALOSE-6-PHOSPHATE PHOSPHATASE
YJL107C	HYPOTHETICAL PROTEIN
CIT2	PEROXISOMAL CITRATE SI -SYNTHASE
YER044C	HYPOTHETICAL PROTEIN
GRE3	HOMOLOGY TO D-XYLOSE 1-DEHYDROGENASE NADP+
YBR051W	QUESTIONABLE ORF
YNL208W	HYPOTHETICAL PROTEIN
LAS1	INVOLVED IN CELL MORPHOGENESIS, CYTOSKELETAL REGULATION AND BUD FORMATION
YOR289W	HYPOTHETICAL PROTEIN
YLR327C	HOMOLOGY TO STF2 PROTEIN
YGR161C	HYPOTHETICAL PROTEIN
AMS1	ALPHA-MANNOSIDASE
YJR061W	SIMILARITY TO YKL200C AND YKL201C
PMC1	CA2+-TRANSPORTING P-TYPE ATPASE
YBR005W	HOMOLOGY TO YDR003W
YOR385W	HOMOLOGY TO HYPOTHETICAL PROTEIN YMR316W
YCR007C	MEMBER OF THE YBR302P/YCR007P/YHL048P/YKL219P FAMILY
YKL159C	HYPOTHETICAL PROTEIN
CMK2	CA/CALMODULIN-DEPENDENT S/T PROTEIN KINASE, TYPE II
PLB1	PHOSPHOLIPASE B LYSOPHOSPHOLIPASE
YMR316W	SIMILARITY TO YOR385W AND YNL165W
YMR007W	HYPOTHETICAL PROTEIN
NUF1	SPINDLE POLE BODY COMPONENT
HSP42	HEAT SHOCK PROTEIN WITH SIMILARITY TO HSP26P
SSA4	HEAT SHOCK PROTEIN
MET6	5-METHYLTETRAHYDROPTEROYLTRIGLUTAMATE-HOMOCYSTEINE METHYLTRANSFERASE
DAN1	SIMILARITY TO PROTEINS OF THE SRP1P/TIP1P FAMILY
TIR1	COLD-SHOCK INDUCED PROTEIN OF THE TIR1P/TIP1P FAMILY
YJR151C	SIMILARITY TO PROTEINS OF THE SRP1P/TIP1P FAMILY
YIL011W	SIMILARITY TO YIL176P,YIR041P AND OTHER MEMBERS OF THE SRP1P/TIP1P FAMILY
YPK2	SER/THR PROTEIN KINASE
CHA1	L-SERINE/L-THREONINE DEAMINASE
ZRT1	HIGH-AFFINITY ZINC TRANSPORT PROTEIN
CTT1	CYTOSOLIC CATALASE T

end of the predicted coding region of the adjacent gene, *YER037w*, is located only 4 bp from the *YER038c* ORF, which had a very similar observed expression pattern (Figure 2). It is therefore likely that the apparent PHO regulation of *YER038c* reflects hybridization to the 3' untranslated region of *YER037w*.

PHM1, PHM2, PHM3, and PHM4 Are Involved in polyP Accumulation

The predicted coding regions Phm1 and Phm2 are similar in length (828 and 835 amino acid residues for Phm1 and Phm2, respectively) and 58% identical in amino acid sequence. The predicted coding region of Phm3 is smaller (648 amino acids) and has 33% identity to the N-terminal region

of Phm1 (Figure 3A). Phm4 is predicted to encode a protein of 129 amino acids, with 32% identity to the C-terminal region of Phm1, which contains three putative transmembrane spanning regions. Phm3 and Phm4 share no homology. Interestingly, the N-terminal regions (amino acids 1–135, Figure 3A) of Phm1, Phm2, and Phm3 have significant similarity to the N-terminal regions of Pho81, and its homolog Ypl110c (Figure 3B). Furthermore, this similarity is shared among a total of nine *S. cerevisiae* ORFs, including the putative phosphate transporter Pho87 (Bun-ya *et al.*, 1996), two proteins homologous to Pho87, Yjl198w and Ynr013, and Syg1 (Spain *et al.*, 1995), a multi-copy suppressor for a *GPA1* deletion. The similarities were confined to the N-terminal region (< 300 amino acids) in all of the nine pro-

Table 2. The PHO regulated genes[a]

ORF	Gene	No. of Pho4 binding site on the promoter		Description[b]
		CACGTG	CACGTT	
Phosphatases				
YBR093c	*PHO5*	1	1	Repressible acid phosphatase (p60)
YAR071w	*PHO11*	1	1	Repressible acid phosphatase (p56)
YHR215w	*PHO12*	1	1	Repressible acid phosphatase (p58)
YDR481c	*PHO8*	1	1	Repressible vacuolar alkaline phosphatase
P_i **transporter**				
YML123c	*PHO84*	2	2	High-affinity H^+/P_i cotransporter
YBR296c	*PHO89*	1	1	Na^+/P_i cotransporter
YJL117w	*PHO86*	1	1	Involved in P_i transport
PHO81 family				
YGR233c	*PHO81*	1	0	Cyclin-dependent kinase inhibitor
YPL110c		1	1	PHO81 homolog
YHR136c	*SPL2*	4	0	PHO81 (ankyrin) homolog
YFL004w family				
YFL004w	*PHM1[c]/VTC2[e]*	1	0	Involved in polyP synthesis[f]
YPL019c	*PHM2[c]/VTC3[e]*	2	1	Homology to YFL004w Involved in polyP synthesis[f]
YJL012c	*PHM3[c]/VTC4[e]*	2	0	Homology to YFL004w Involved in polyP synthesis[f]
YER072w	*PHM4[c]/VTC1[e]/NRF1[e]*	1	1[d]	Homology to YFL004w Involved in polyP synthesis[f] Vacuolar transporter chaperon[e]
Others				
YER062c	*HOR2/GPP2*	1	0	DL-glycerol phosphatase
YPL018w	*CTF19*	2	1	Important for chromosome segregation
YER055c	*HIS1*	1	0	ATP phosphoribosyltransferase
YDR452w	*PHM5[c]*	1[d]	0	Similar to acid sphingomyelinase Supposed vacuolar polyphosphatase[f]
YDR281c	*PHM6[c]*	1	2	Hypothetical protein
YOL084w	*PHM7[c]*	1[d]	0	Similarity to *S. pombe* SPAC24H6.13 and YMR266w
YER037w	*PHM8[c]*	0	1	Similarity to hypothetical protein YGL224c
YER038c		0	0	Function unknown

[a] The genes listed in this table showed more than two-fold derepression ratio in more than five independent experiments out of the eight in Figure 2 except for *YGR233c (PHO81)* and *YPL110c*.

[b] Description taken from Yeast Proteome Database *http://www.proteome.com/*.

[c] *PHM1-8* are novel genes, named in this study.

[d] 5/6 nucleotide sequences match to the Pho4 binding sequences.

[e] *VTC1–VTC4* and *NRF1* were independently named and characterized by Cohen *et al.*, (1999) and Murray and Johnson (2000), respectively, in course of this study.

[f] These functions were identified in this study.

teins. Homologous sequences can also be found in the databases of *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, mouse, and human (Battini *et al.*, 1999).

To address the function of the *PHM1*, *PHM2*, *PHM3*, and *PHM4* genes, strains carrying deletions of each of these genes were constructed (see MATERIAL AND METHODS). All five mutant strains, including a *phm1Δ phm2Δ* double mutant, were viable in rich media (YPAD), and no significant growth defects were observed in low-P_i synthetic media. All five were able to produce acid phosphatases in response to low P_i. When we analyzed polyP accumulation, however, the mutants showed striking phenotypes.

It is known that *S. cerevisiae* accumulates a large amounts of polyP in vacuoles under conditions of high P_i preceded by a period of P_i starvation. This is referred to as the "polyphosphate overplus" phenomenon (Harold, 1966). PolyP chains in extracts from yeast were analyzed by PAGE followed by staining with a Toluidine Blue dye, which stains polyP as well as nucleic acids. The extract from wild-type cells, NBW7, after the polyP overplus treatment (see MATERIAL AND METHODS) resulted in two distinct populations of stained molecules as is shown in Figure 4A. The upper population represents RNA, whereas the lower "ladder-like" population is polyP, confirmed by pretreatment with either RNase A or ex-
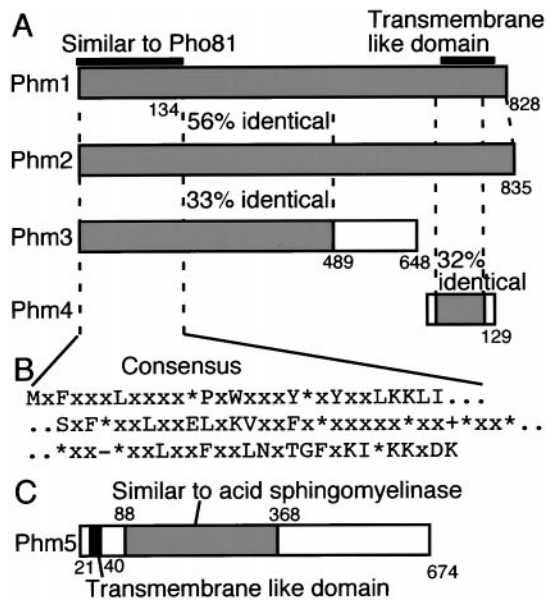
Figure 3. (A) Homology between Phm1, Phm2, Phm3, and Phm4. Shaded and white boxes represent homologous and nonhomologous regions, respectively, and their identities to the Phm1 sequence are indicated by percentage. The region homologous to Pho81 and the putative transmemembrane region are indicated above the Phm1 box. (B) Consensus sequences in the N-terminal regions of nine *S. cerevisiae* proteins, including Pho81. Hydrophobic, positive- and negative-charged amino acid residues are indicated by "*," "+," and "-," respectively. (C) The putative Phm5 protein structure. The region of sequence similarity to acid sphingomyelinase in human and *C. elegans* is indicated by a shaded box. The putative transmembrane region is indicated by a filled box.

Figure 4. PolyP analysis by PAGE for various *phm* mutants. Each polyP sample, containing 10-μg RNA, prepared from indicated strains after the polyP overplus culture, was loaded on 20% polyacrylamide gel, and subjected to electrophoresis at 20 V/cm for 1.5 h (panel A) or 3 h (panel B). The strains used were NBW7 (WT), NBM4Lf1 (*phm1Δ*), NBM19Hf1 (*phm2Δ*), NBM4L19H2 (*phm1Δ phm2Δ*), NBM12W2 (*phm3Δ*), NBM72W7 (*phm4Δ*), NBM452H1 (*phm5Δ*), NBM18H7 (*ctf19Δ*), NBM281H1 (*phm6Δ*), NBD4–1 (*pho4Δ*), NBD80–1 (*pho80Δ*), and NBD8184–1 (*pho84Δ*). Several RNA bands are visible in a region near the top of the both gels, indicated by "RNAs". Marker samples containing 10 μg of sodium phosphate glass type 5, 15, and 35 were loaded on the P5, P15, and P35 lanes, respectively. Extracted polyP samples corresponding to 55 and 65 P_i residues were also loaded as size markers (PP55 +65). Chain lengths of the polyP ladders determined by the markers (Materials and Methods) are indicated to the left and right of panel A and B, respectively. The migration positions indicated with the chain length in the parenthesis on panel B are estimated from a standard curve.

opolyphosphatase (our unpublished results). The chain lengths of the polyP bands were determined by comparing them with polyP marker ladders run in a nearby lane. The distribution of the polyP chain length in the NBW7 strain was broad (~100 P_i residues) with a median length of ~ 60 P_i residues (Figure 4B). The cellular concentration was measured at 19.4 μmol (1.55 mg) of polyP/mg total RNA by an enzymatic assay (Ault-Riché *et al.*, 1998) (Table 3).

The effects of *PHM1-PHM4* on polyP accumulation following the polyP overplus treatment were analyzed by gel electrophoresis (Figure 4) and enzymatic assay (Table 3). Total polyP was slightly reduced in the *phm1Δ* mutant. The *phm2Δ* mutant had significantly reduced total polyP (14% of wild-type), and the median size of the polyP molecules was significantly smaller than that in the wild-type. PolyP was undetectable in the *phm1Δ phm2Δ* double mutant. This clearly demonstrates that the *PHM1* and *PHM2* genes have redundant functions in polyP accumulation. Both the *phm3Δ* and *phm4Δ* mutants lacked detectable polyP. These results suggest that Phm1, 2, 3 and 4 proteins are involved in the accumulation of polyP and that the products of Phm3, Phm4 and either Phm1 or Phm2 are required for polyP accumulation.

Phm2 is Localized to the Vacuole

More than 90% of total polyP in yeast is localized to the vacuole (Urech, 1978). Since the Phm1–Phm4 proteins are involved in polyP synthesis, we speculated that the Phm1–Phm4 proteins were also localized to the vacuole. To address this question, a *PHM2-GFP* fusion gene, which encodes the full *PHM2* ORF fused to the *GFP* ORF, was constructed and

Table 3. PolyP amounts in the various mutants

Strain	Mutation	PolyP (μmol/mg of RNA)
NBW7	wild type	19.4
NBM-4Lf1	*phm1Δ*	11.3
NBM-19Hf1	*phm2Δ*	2.77
NBM-4L19H2	*phm1Δ phm2Δ*	<0.01
NBM-12W2	*phm3Δ*	<0.01
NBM-72W7	*phm4Δ*	<0.01
NBM-452H1	*phm5Δ*	12.4
NBM-18H7	*ctf19Δ*	9.73
NBM-281H1	*phm6Δ*	8.93
NBD4-1	*pho4Δ*	0.417
NBD80-1	*pho80Δ*	<0.01
NBD8184-1B	*pho84Δ*	0.015

expressed in wild-type and *phm1Δ phm2Δ* double mutant strains. The fusion protein was active; *PHM2-GFP* was able to complement the polyP deficient phenotype of the *phm1Δ phm2Δ* mutant (our unpublished results). Phm2-GFP indeed appeared to be localized to the vacuoles (Figure 5) by colocalization with the vacuolar membrane marker FM4–64 (Vida and Emr, 1995).

While this study was in progress, Cohen *et al.* (1999) reported on a gene family involved in a vacuolar transporter chaperon, *VTC1*, *VTC2*, *VTC3*, and *VTC4* in *S. cerevisiae*. These four genes are identical to *PHM4*, *PHM1*, *PHM2*, and *PHM3*, respectively. In their report, the Phm4/Vtc1 protein was originally found in the same fraction as a Vma10 protein, a subunit of vacuolar H$^+$-ATPase (v-ATPase) localized on the vacuolar membrane. Furthermore, Phm3/Vtc4, which does not have a putative transmembrane domain, was found in the membrane fraction only in the presence of Phm4/Vtc1 and either Phm1/Vtc2 or Phm2/Vtc3. These

Figure 6. (A) PolyP PAGE analysis of *vma4* and *pep4* mutants. PolyP samples were prepared from the strains, CRY-V4 (*vma4Δ*), CRY (WT), CRX (*ppx1Δ*), CB024 (Pro), and NB91–6A (*pep4*) cultivated by the polyP overplus method. The other conditions were as described in Figure 4A. (B) PolyP PAGE analysis of *vma4* and *phm* mutants cultivated at low pH. PolyP samples were prepared from the strains, CRY (WT), CRY-V4 (*vma4Δ*), NBM4L19H2 (*phm1Δ phm2Δ*), NBM12W2 (*phm3Δ*), and NBM72W7 (*phm4Δ*) cultivated in YPAD-Pi media, followed by cultivation in YPAD supplemented with either 10 mM P$_i$ (P$_i$), or 10 mM P$_i$ and 10 mM sodium acetate buffer (Ac), for 2 h. The other conditions were as described in Figure 4A.

Figure 5. Localization of Phm2-GFP. The identical frames of the yeast transformant NBM4L19H1[pPHM2-GFP] were shown for Normarsky (left column), FM 4–64 fluorescence(center column), and GFP fluorescence (right column) images.

finding support our hypothesis that the Phm1 through 4 proteins form a complex on the vacuolar membrane. In addition, an *S. pombe* protein, Nrf1, with an amino acid sequence 78% identical to that of Phm4/Vtc1, was recently reported as a vacuolar membrane protein (Murray and Johnson, 2000).

PolyP Synthesis Is Influenced by v-ATPase Activity but It Is Not Essential

Previous work has shown that polyP accumulation is dependent upon v-ATPase activity (Wurst *et al.*, 1996). A *vma4* mutant strain, in which v-ATPase activity is completely deficient (Zhang *et al.*, 1998), was reproducibly found to have no accumulation of polyP under the polyP overplus conditions (Figure 6A). Cohen *et al.*, (1999) reported that the
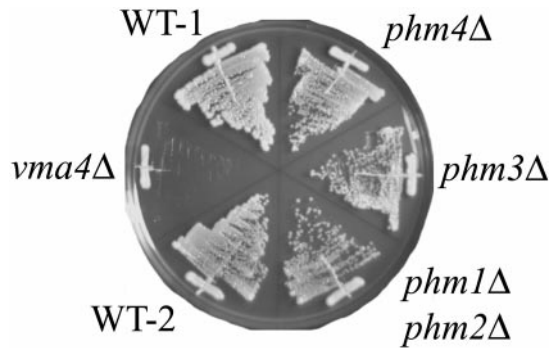
Figure 7. Ca^{2+} sensitivities of *phm* mutants at neutral pH. CRY (WT-1), CRY-vma4 (*vma4Δ*), NBW7 (WT-2), NBM4L19H2 (*phm1Δ phm2Δ*), NBM12W2 (*phm3Δ*), and NBM72W7 (*phm4Δ*) were streaked on the YPAD plate (pH 7.5 supplemented with 60 mM CaCl$_2$, and incubated at 30°C for 3 days.

Figure 8. P$_i$ uptake in *phm* mutants. P$_i$ uptake activities in the strains NBW7 (WT), NBM4Lf1 (*phm1Δ*), NBM19Hf1 (*phm2Δ*), NBM4L19H2 (*phm1Δ phm2Δ*), NBM12W2 (*phm3Δ*), NBM72W7 (*phm4Δ*), and NBD8184–1B (*pho84Δ*) were tested as described in Materials and Methods.

v-ATPase activity in a *phm4Δ/vtc1Δ* mutant was 10–30% of that in wild-type cells. Cells with mutations in v-ATPase are characteristically deficient in respiration and sensitive to media containing 60 mM CaCl$_2$ at pH 7.5 (Zhang *et al.*, 1998). To address the possibility that the Phm1 through 4 proteins could be necessary for v-ATPase activity, and thus indirectly for the accumulation of polyP, the *phm* disruptants were tested for growth on nonfermentable carbon sources and in the presence of CaCl$_2$. Growth of the *phm1Δ phm2Δ*, *phm3Δ*, and *phm4Δ* mutants in 60 mM CaCl$_2$ was similar to that of the wild-type strain (Figure 7), whereas the *vma4* mutant did not grow. Moreover, *phm1Δ phm2Δ*, *phm3Δ*, and *phm4Δ* mutants were able to grow on rich media with ethanol or glycerol as a sole carbon source, while the *vma4* mutant was not (our unpublished results). These data suggest that the *phm* mutants retain v-ATPase activity.

It is known that incubating yeast in low pH media can, in part, reverse the phenotype seen with v-ATPase mutants by lowering the intravacuolar pH via fluid-phase endocytosis (Nelson and Nelson, 1990). A low but significant level of polyP accumulation was detected when the *vma4* mutant was incubated at low pH (Figure 6B). In contrast, the *phm1Δ phm2Δ*, *phm3Δ* and *phm4Δ* mutants failed to accumulate detectable polyP with the low pH incubation (Figure 6B). These results strongly suggest that the *PHM1-PHM4* gene products are directly involved in polyP accumulation and that v-ATPase activity is not strictly essential for polyP synthesis.

PolyP Formation Prevents Short-term Saturation of Cellular P$_i$ Accumulation

Phm1 through 4 proteins clearly play a role in polyP synthesis. The apparently paradoxical increase in the cell's ability to convert P$_i$ into polyP in response to P$_i$ starvation might represent a strategy for accumulating and holding precious P$_i$. We therefore measured P$_i$ uptake in the *phm1-phm4* mutants (Figure 8). After cultivation in P$_i$-free synthetic media for 2h, NBW7 wild-type cells displayed a linear (nonsaturable) P$_i$ uptake for up to 25 min when incubated in 0.1 mM P$_i$ (twofold less concentration of synthetic low-P$_i$ medium). In contrast, the *pho84Δ* mutant, which is deficient in H$^+$/P$_i$
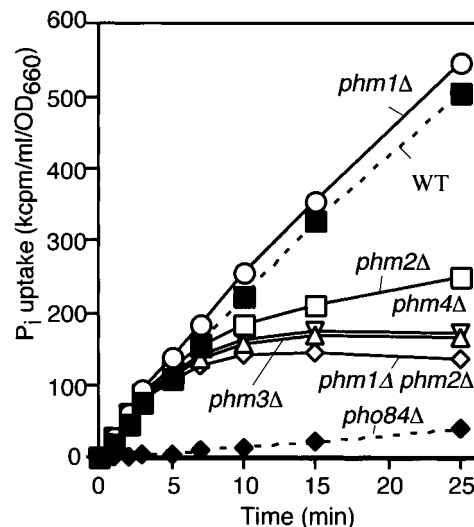
cotransporter activity, showed very low uptake, as reported previously (Bun-ya *et al.*, 1991). The *phm1, 2, 3,* and *4* mutants each had unique saturable P$_i$-uptake profiles. The *phm1Δ* single mutant had an uptake profile similar to wild-type. The *phm2Δ* single mutant showed a rate of uptake similar to wild-type from 0 to 8 min, but after 8 min uptake was minimal. The P$_i$-uptake profiles of the *phm1Δ phm2Δ* double mutant, the *phm3Δ*, and the *phm4Δ* mutants were similar to wild-type for the first 5 min, after which uptake appeared to cease. This cessation of uptake in the *phm* mutants correlated well with the overall ability of each mutant to accumulate polyP. These data suggest that polyP accumulation is required, presumably as a sink, to sustain a high rate of P$_i$ uptake.

PHM5 May Encode a Polyphosphatase in the Vacuole

The previously uncharacterized ORFs *YDR452w*, *YDR281c*, *YOL084w*, and *YER037w* were also found to be PHO-regulated. They are therefore named *PHM5* through *PHM8*, respectively. *YDR281c/PHM6* and *YER037w/PHM8* were previously noted by Gray *et al.* (1998) to be induced by treatment with drugs that inhibit Cdc28 and Pho85 kinases. Among those four genes, Phm5 has significant homology to human and *C. elegans* acid sphingomyelinases (Figure 3C) which function in hydrolyzing phosphodiester bonds in sphingomyelin. When *phm5Δ*, *ctf19Δ*, and *phm6Δ* single disruptants were constructed and tested for polyP accumulation levels, we observed that the *phm5Δ* mutant had a unique distribution of longer chain length polyP when compared with wild-type (Figure 4A). PolyP levels and size in the *ctf19Δ* and *phm6Δ* mutants were indistinguishable from wild-type. The average polyP chain length in the *phm5Δ* mutant was over 150 P$_i$ residues (Figure 4B). By enzymatic

measurement, the total amount of polyP in the *phm5Δ* mutant was similar to that of wild-type (Table 3), indicating that polyP synthesis activity in *phm5Δ* was unaffected. These data suggest that the Phm5 protein is associated with, or is a polyphosphatase.

The deduced amino acid sequence of Phm5 (Figure 3C) includes a putative N-terminal membrane spanning domain, similar in size (27 amino acids), position and flanking amino acid sequences (rich in serine and positive charged residues) to that of the Pho8 protein, a PHO-regulated vacuolar alkaline phosphatase, which is anchored to the vacuolar membrane by a transmembrane domain at its N-terminus (Klionsky and Emr, 1989).

Wurst *et al.* (1995) reported that a mutant deficient in three vacuolar proteinases: proteinase A, proteinase B, and carboxypeptidase Y, has a longer polyP chain length distribution than the wild-type. This phenotype was reproducibly observed in our PAGE assay (Figure 6A). Furthermore, the phenotype was observed in a *pep4* single mutant, which has a defect only in Proteinase A, which is required for maturation of several vacuolar enzymes, including Pho8. Together with Phm5's similarity to sphingomyelinases, these data suggest that *PHM5* encodes a polyphosphatase, which is matured by the vacuolar proteinase. Kumble and Kornberg (1996) purified a processed endopolyphosphatase of 35 kDa from yeast. Recently, it has been found that the 35-kDa protein contains amino acid sequences identical to the deduced Phm5 sequence (Sethuraman and Kornberg, personal communication).

Mutations in the PHO Regulator Genes Affect polyP Accumulation

Since the genes for polyP processing are regulated by the PHO regulatory system, polyP accumulation should be affected by mutations which affect this system. To investigate this, polyP levels under polyP overplus conditions were measured in *pho4Δ*, *pho80Δ*, and *pho84Δ* mutants (Figure 4A). The *pho4* mutant, which is incapable of derepression of its target genes (Figure 1) accumulates a lower level of polyP than wild-type. The *pho84Δ* mutant, lacking a high-affinity P_i transporter, accumulates a very low but detectable level of polyP. The *pho80Δ* mutant, surprisingly, did not accumulate detectable levels of polyP, despite the fact that *PHO84,* and *PHM1* through *4* are highly expressed in this mutant. This paradoxical phenotype may be a consequence of an abnormal vacuole in this mutant (Nicolson *et al.*, 1995).

DISCUSSION

The Proteins Involved in the P_i Acquisition and Storage System in Yeast

Using DNA microarray technology, we have identified at least 22 genes, including 13 novel genes, whose expression is regulated by the PHO pathway. Based on the premise that PHO-regulated genes are integral components of P_i metabolism, further molecular genetic approaches were undertaken to search for the function of the previously-uncharacterized genes. Our work has identified five of these genes as being involved in polyP metabolism. Thus, a total of 17 genes have now been found to function in P_i acquisition in yeast (Figure 9).
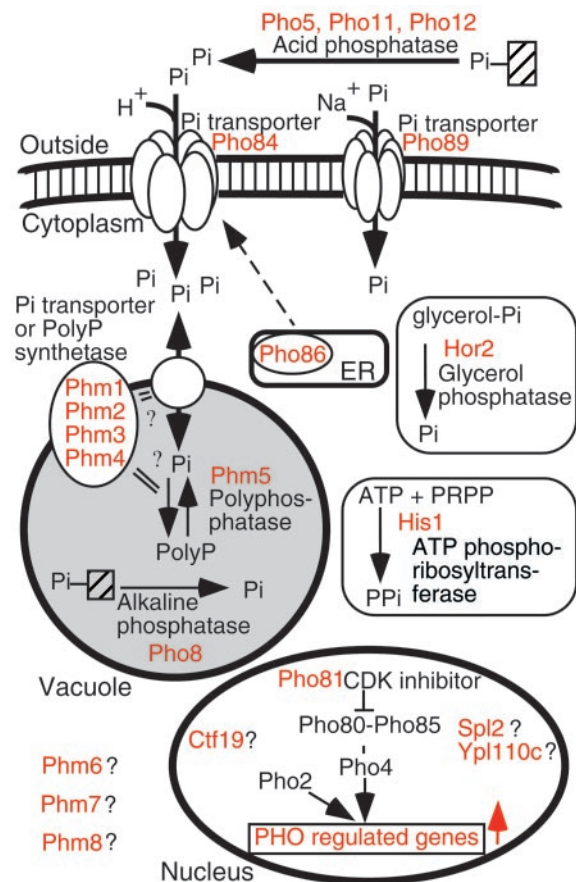

Figure 9. P_i acquisition and storage system in *S. cerevisiae*. The known of predicted roles of proteins encoded by the PHO-regulated genes (names indicated in red) are schematized. See text for details.

It is interesting to consider how the products of those 17 genes work together as a physiological system for P_i acquisition and storage. When yeast encounter conditions of P_i starvation, the low P_i signal initiates Pho81 activity, which suppresses Pho80-Pho85 kinase activity. The ankyrin domain of the Pho81 protein is sufficient to inhibit Pho80-Pho85 activity (Schneider *et al.*, 1994; Ogawa *et al.*, 1995). The PHO-regulated Pho81 homologs *YPL110c* and *SPL2* share this domain, but their involvement in Pho80-Pho85 inhibition remains obscure (Flick and Thorner, 1998). Inhibition of the Pho80-Pho85 kinase results in an active Pho4 protein (hypophosphorylated form), which is localized to the nucleus where it acts as a specific transcriptional activator of PHO-regulated genes. Transcribed genes include *PHO81*, providing a positive feedback loop, which acts to keep Pho4 in its active form (Ogawa *et al.*, 1995), resulting in high, continued expression of all PHO-regulated genes.

The P_i starvation signal triggers increased production of at least four types of phosphatases; 1) the acid phosphatases Pho5, Pho11, and Pho12, which are localized in periplasmic space; 2) the alkaline phosphatase Pho8, which is localized to the vacuole, 3) the glycerol phosphatase Hor2; 4) the putative polyphosphatase Phm5, localized in the vacuole. The acid and alkaline phosphatases are nonspecific, and

hydrolyze a variety phosphorylated substrates, including nucleic acids, phosphosugars, phospholipids, and phosphoproteins. The glycerol phosphatase hydrolyzes the phosphate ester bond of glycerolphosphate, which is found in many sugar and lipid metabolites. All of these enzymes can contribute to increased levels of free P$_i$.

Under conditions of P$_i$ starvation, expression of genes encoding the phosphate transporters, Pho84 and Pho89, are induced. Their optimal conditions are quite different: Pho84 transports P$_i$ optimally at pH 4 and cotransports H$^+$. Pho89, on the other hand, has optimal activity at pH 9.0 and cotransports Na$^+$. This pair of P$_i$ transporters work well in a wide range of environmental conditions in which yeast live. Expression of *PHO86*, a P$_i$ transporter-related gene, is also increased in P$_i$ starvation. The Pho86 protein was originally thought to form a complex with Pho84 and Pho87 (Bun-ya *et al.*, 1996), however Lau *et al.* (2000) recently found that Pho86 is localized to the endoplasmic reticulum, where it functions in the proper localization of the Pho84 protein to the plasma membrane. Thus, Pho86 is now thought to act indirectly in P$_i$ uptake.

The *PHM1* through *4* genes, which we have shown to be involved in polyP synthesis, contribute to the P$_i$ accumulation by a unique mechanism. Our results suggest that polyP synthesis is required for proper P$_i$ accumulation. When polyP synthesis is critically slow, it can control the rate at which P$_i$ is taken up by Pho84 membrane transporter. When polyP synthesis is slow, intracellular free P$_i$ levels become high, which in turn acts as a direct negative feed back on the Pho84 membrane transporter. This critical intracellular P$_i$ level is achieved after approximately 5 min of incubation in media containing 0.1 mM P$_i$. The *phm* mutants that lacked detectable polyP synthesis activity (*phm1Δ phm2Δ* double, *phm3Δ* and *phm4Δ* single disruptants) showed rapid initial uptake of P$_i$ (like wild-type) but were incapable of further P$_i$ uptake after ~ 5 min. Interestingly, the *phm2* mutant, which had some residual polyP synthesis activity (~ 10% of wild-type) could continue to accumulate P$_i$ after the initial 5-min period, but did so at a very reduced rate. In this mutant, it appears that the rate-determining step in P$_i$ uptake in the first 5 min was controlled by the Pho84 membrane transporter, and after this time the rate was controlled by P$_i$ to polyP conversion.

In this study, we have shown that polyP plays an important role in P$_i$ accumulation and metabolism in yeast. The evidence for this involvement is not only metabolic but also genetic. Similar genetic interactions between polyP and P$_i$ metabolism have been observed in *E. coli*. The promoter of the *ppk-ppx* operon, containing the genes for polyP kinase and exopolyphosphatase, includes a *pho* box, the response element for the P$_i$ starvation signal mediated by the *phoB-phoR* two-component regulator (Kato *et al.*, 1993). This suggests that the corresponding bacterial genes are regulated in a manner analogous to *PHM1* through *4* and *PHM5*, respectively. *Vibrio cholerae*, a Gram-negative bacterium, has a similar *ppk-ppx* operon, with a *pho* box in its promoter, and its *ppk* mutant was unable to sustain a high rate of P$_i$ accumulation (Ogawa *et al.*, 2000). Thus, regulation by P$_i$ appears to be a physiologically conserved feature of the genes for polyP metabolism, in both bacteria and yeast. Moreover, these results suggest that a major physiological role of polyP may be to promote long-term uptake and accumulation of P$_i$.

PHM5 Encodes a Vacuolar Polyphosphatase

Prior to this study, the *PPX1* gene, encoding an exopolyphosphatase, was the only yeast gene implicated in polyP processing (Wurst *et al.*, 1995). The expression of the *PPX1* gene in this study showed no detectable response to P$_i$ levels or perturbation of PHO regulation. Ppx1 protein is believed to be localized to the cytoplasm, which contains negligible amounts of polyP. Since > 90% of total cellular polyP is accumulated in vacuoles (Urech *et al.*, 1978), the principal physiological polyphosphatase is likely to be vacuolar. The predicted vacuolar localization and PHO-regulation of Phm5 suggest that it is likely to contribute significantly to polyP degradation in vivo. Indeed, the *phm5* mutation resulted in a marked increase in polyP chain lengths, whereas the *ppx1* mutation resulted in a much smaller change (Figure 6A).

Phm1 through 4 Proteins Represent a New Type of polyP Synthesis System

Every Gram-negative bacterium for which the genome has been sequenced, has genes homologous to *E. coli* PPK (Tzeng and Kornberg, 1998). No genes homologous to PPK have been found in the genomes of Gram-positive bacteria, archea, or eukaryotes. This PPK is the only reported enzyme capable of synthesizing polyP, despite the fact that polyP has been found in every organism in which it has been sought, including Gram-positive bacteria, eukaryotes, and archea (Kornberg, 1999). Kornberg (1999) has speculated that mammalian cells synthesize polyP directly from the incorporated P$_i$ without an ATP intermediate. Our results suggest that eukaryotic cells have an enzyme system for polyP synthesis completely different from the PPK of Gram-negative bacteria. PolyP synthesis in yeast requires v-ATPase activity, which produces proton motive force across the vacuolar membrane. We therefore speculate that the high-energy phosphoanhydride bonds in polyP are directly synthesized from P$_i$, using the proton motive force as a source of energy, by a vacuolar membrane-bound enzyme(s), analogous to the F-type ATPase in mitochondria. Based on this hypothesis, the Phm1 through 4 protein complex is the best candidate for this polyP synthesis enzyme in yeast.

Does a P$_i$ acquisition system similar to the yeast system exist in higher eukaryotic cells? Kido *et al.* (1999) reported that expression of type II Na$^+$/P$_i$ cotransporter gene, *NPT2*, in rat kidney is derepressed by a dietary P$_i$ starvation, and that the P$_i$ signal-responsive element in its promoter contains the sequence, CACGTG, which is identical to the Pho4-binding site in yeast. Moreover, the N-terminal regions of Phm1, Phm2, and Phm3 contain conserved domains shared with six additional *S. cerevisiae* proteins, and similar protein structures can be found in genomes of many eukaryotes. Eight of the nine yeast proteins with this domain are related to P$_i$ metabolism. We therefore refer to this domain as the "phosphate (P$_i$) domain" (Figure 3B). Since most of the homologous genes in other species have little or no functional characterization (except for human XPR1, gene encoding xenotropic and polytropic retrovirus receptor [Battini *et al.*, 1999]), the function of the P$_i$ domain may provide a useful lead for further functional investigations. Thus, further studies of the yeast system for P$_i$ metabolism are likely

to provide fundamental insights into P_i metabolism in all eukaryotes.

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