A Genetic Study of Signaling Processes for Repression of *PHO5* Transcription in *Saccharomyces cerevisiae*

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, transcription of a secreted acid phosphatase, *PHO5*, is repressed in response to high concentrations of extracellular inorganic phosphate. To investigate the signal transduction pathway leading to transcriptional regulation of *PHO5*, we carried out a genetic selection for mutants that express *PHO5* constitutively. We then screened for mutants whose phenotypes are also dependent on the function of *PHO81*, which encodes an inhibitor of the Pho80p-Pho85p cyclin/cyclin-dependent kinase complex. These mutations are therefore likely to impair upstream functions in the signaling pathway, and they define five complementation groups. Mutations were found in a gene encoding a plasma membrane ATPase (*PMA1*), in genes required for the *in vivo* function of the phosphate transport system (*PHO84* and *PHO86*), in a gene involved in the fatty acid synthesis pathway (*ACC1*), and in a novel, nonessential gene (*PHO23*). These mutants can be classified into two groups: *pho84*, *pho86*, and *pma1* are defective in high-affinity phosphate uptake, whereas *acc1* and *pho23* are not, indicating that the two groups of mutations cause constitutive expression of *PHO5* by distinct mechanisms. Our observations suggest that these gene products affect different aspects of the signal transduction pathway for *PHO5* repression.

ALL cells must respond appropriately to changes in their environment. When microorganisms are limited for nutrients, they respond by regulating expression of genes important for survival. In the yeast *Saccharomyces cerevisiae*, transcription of a gene for a secreted acid phosphatase, *PHO5*, is regulated in response to changes in the extracellular concentration of inorganic phosphate. *PHO5* transcription is repressed in high-phosphate medium and derepressed in low-phosphate medium.

A genetic pathway for *PHO5* regulation has been established by Oshima and colleagues (Oshima 1982). *PHO2*, *PHO4*, and *PHO81* are positive regulators of *PHO5*; deletion of these genes results in an inability to induce *PHO5* upon phosphate starvation (Toh-e *et al.* 1973). Another set of genes, including *PHO80*, *PHO85*, and *PHO84*, are required for *PHO5* repression, and loss-of-function mutations in these genes result in constitutive expression of *PHO5*, even in high-phosphate medium (Pho^c phenotype; Ueda *et al.* 1975).

In recent years, progress has been made in elucidating the molecular mechanism of a signal transduction pathway leading to the transcriptional regulation of *PHO5* (Lenburg and O'Shea 1996). *PHO4* encodes a transcription factor that is required for *PHO5* expression (Toh-e *et al.* 1973). When yeast cells are grown in high-phosphate medium, Pho4p is phosphorylated by the Pho80p-Pho85p cyclin/cyclin-dependent kinase (CDK) complex (Kaffman *et al.* 1994). Phosphorylated Pho4p is localized predominantly to the cytoplasm (O'Neill *et al.* 1996), and *PHO5* transcription is repressed. When yeast cells are starved for phosphate, the activity of the Pho80p-Pho85p cyclin-CDK complex is inhibited by the CDK inhibitor (CKI) Pho81p (Schneider *et al.* 1994; Ogawa *et al.* 1995). Under these conditions, Pho4p is unphosphorylated and localized to the nucleus, where it activates *PHO5* transcription.

In high-phosphate medium, repression of *PHO5* transcription requires inactivation of the CKI activity of Pho81p. However, it is still not known where the signal to repress *PHO5* transcription is generated, nor how the signal results in activation of the kinase activity of the Pho80p-Pho85p cyclin-CDK complex. Loss-of-function mutations in *PHO81* are epistatic to mutations in *PHO84*, suggesting that *PHO84* functions upstream of *PHO81*. Biochemical data are consistent with this model—Pho84p is a transmembrane protein required *in vivo* for high-affinity phosphate uptake (Tamai *et al.* 1985; Bun-Ya *et al.* 1991), and recombinant Pho84p expressed in *Escherichia coli* is capable of transporting phosphate *in vitro* when assembled into synthetic phospholipid vesicles (Berhe *et al.* 1995). Whether Pho84p is directly involved in the signaling that leads to *PHO5* repression is currently unclear.

In an effort to understand the signaling process for *PHO5* repression, we designed a genetic selection using a *PHO5-HIS3* reporter to identify genes that function

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upstream of *PHO81* and are required for *PHO5* repression. Our strategy was to isolate mutants that exhibit a Pho^c phenotype that is also *PHO81* dependent. Our goal was to identify components of the signal transduction pathway required to prevent inhibition of the Pho80p-Pho85p complex by Pho81p in high-phosphate medium. Loss-of-function mutations in these genes should cause accumulation of unphosphorylated Pho4p in the nucleus in high-phosphate medium as a result of inhibition of Pho80p-Pho85p kinase activity by Pho81p. We have determined that mutations in five genes (*PHO84*, *PHO86*, *PMA1*, *ACC1*, and *PHO23*) result in constitutive *PHO5* expression in a *PHO81*-dependent manner. Mutations in *PMA1*, *ACC1*, and *PHO23* have not been reported to confer a Pho^c phenotype. Analysis of these mutants indicates that these genes are likely to affect different aspects of the signaling pathway. Possible mechanisms for the action of these gene products in the phosphate repression signaling pathway are discussed.

MATERIALS AND METHODS

Yeast strains, plasmids, media, and genetic methods: Yeast strains used in this study are described in Table 1. Our yeast strains are derived from *K699* or have been crossed into the *K699* genetic background (Schwob and Nasmyth 1993). Plasmids are listed in Table 2. Standard yeast media are as described (Ausubel *et al.* 1993). Low-phosphate medium is phosphate-depleted medium (O'Connell and Baker 1992). No-phosphate medium is synthetic medium consisting of yeast nitrogen base lacking inorganic phosphate. Yeast nitrogen base lacking inorganic phosphate was made with components described in the Difco manual, except that potassium phosphate was substituted with the same amount of potassium chloride. Uptake medium consists of no-phosphate medium containing 10 mm potassium acetate to buffer it to pH 4.2

TABLE 1

Yeast strains

Strain	Relevant genotype	Source or reference
EY0100	*MAT*α *his3::pPHO5-HIS3*[a]	This work
EY0183	*MAT***a** *lys2Δ99*	This work
EY0134	*MAT*α *pho80Δ::HIS3*	E.K.O. collection
EY0211	*MAT***a** *lys2Δ99 pho84Δ::HIS3*	This work
EY0179	*MAT***a** *lys2Δ99 pho80Δ::HIS3 pho85Δ::LEU2*	This work
EY0150	*MAT***a** *pho81Δ::TRP1*	E.K.O. collection
EY0174	*MAT*α *pho80Δ::HIS3 pho81Δ::TRP1*	This work
EY0152	*MAT***a** *pho84Δ::HIS3 pho81Δ::TRP1*	This work
EY0404	*MAT***a** *pho86Δ::TRP1*	E.K.O. collection
EY0452	*MAT*α *acc1-29*	This work
EY0453	*MAT*α *pho86-153*	This work
EY0454	*MAT*α *pma1-196*	This work
EY0455	*MAT*α *pho23-1*	This work
EY0456	*MAT***a** *lys2Δ99 pho23Δ::HIS3*	This work
EY0457	*MAT***a** *lys2Δ99 acc1-29 pho81Δ::TRP1*	This work
EY0458	*MAT***a** *lys2Δ99 pho86-153 pho81Δ::TRP1*	This work
EY0459	*MAT***a** *lys2Δ99 pma1-196 pho81Δ::TRP1*	This work
EY0460	*MAT***a** *lys2Δ99 pho23-1 pho81Δ::TRP1*	This work
EY0464	*MAT***a** *lys2Δ99 acc1-29 pma1-196*	This work
EY0465	*MAT***a** *lys2Δ99 acc1-29 pho23Δ::HIS3*	This work
EY0466	*MAT***a** *lys2Δ99 pma1-196 pho23Δ::HIS3*	This work
EY0468	*MAT***a** *lys2Δ99 ACC1::acc1-URA3*	This work
EY0469	*MAT***a** *lys2Δ99 PMA1::PMA1-LEU2*	This work
EY0486	*MAT***a** *pho80Δ::TRP1*	E.K.O. collection
HKY479-2A	*MAT*α *ade2-1 trp1-1 can 1-100 leu2-3, 112 his3-11, 15 ura3 acc1cs*	Guerra and Klein (1995)
YRXS11	*MAT***a** *ade2-201 leu2-Δ1 ura3-52 GAL+ lys2801 acc1-7-1*	Schneiter *et al.* (1996)
acc1-2150	*MAT*α *acc1-2150*	Mishina *et al.* (1980)
acc2-3826	*MAT***a** *acc2-3826*	Mishina *et al.* (1980)
JWY270	*MAT*α *ras2Δ::LEU2*	J. Whistler and J. Rine
ΔΔH285Q	*MAT*α *pma1Δ::HIS3 pma2Δ::TRP1 lys2-801 ade2-101 ura3-52* [p*pma1-H285Q LEU2 CEN6 ARSH4*]	Wach *et al.* (1996)
MG2129	*MAT*α *pma1-A608T*	van Dyck *et al.* (1990)
I183A	*Y55 HO pma1-I183A*	Wang *et al.* (1996)
S368F	*Y55 HO pma1-S368F*	Perlin *et al.* (1989)
A135V	*Y55 HO pma1-A135V*	Na *et al.* (1993)
ACY7	*pma1-7 (P434A and G789S)*	Chang and Fink (1995)

[a] All strains from this lab are *K699 ade2-1 trp1-1 can 1-100 leu2-3, 112 his3-11,15 ura3 GAL+*.

TABLE 2

Plasmids

Plasmid	Description	Source
EB0657	*ACC1* ORF in YCp50; isolated from a genomic library	This work
EB0658	*Xho*I/*Hin*dIII fragment from *PMA1* in the *Sal*I/*Hin*dIII sites of YCp50	This work
EB0659	*Pst*I/*Spe*I fragment from *PH023* (*YNL097c*) in pRS314	This work
EB0660	*Pst*I/*Xho*I fragment from *PMA1* in pRS305	This work
EB0661	*Hin*dIII fragment from *ACC1* in pRS306	This work
EB0662	Disruption vector for *PHO23* marked with *HIS3*	This work
EB0180	*PHO5* promoter fused in frame to the N terminus of GFP in pRS316	EKO collection
EB0347	*PHO4* promoter and the entire *PHO4* ORF fused in-frame to N terminus of GFP in pRS316	EKO collection
pMB220	*PHO86* in YCp50	Yompakdee *et al.* (1996)
pJL428	*LYS2* disruption vector	Gift from Joachim Li

or 10 mm potassium citrate to buffer it to pH 3.0. Media supplemented with fatty acids were prepared by adding 1% Tween 40 (v/v) with 0.05% (w/v) palmitic acid. Crossing, sporulation, and tetrad analysis were done by standard genetic methods (Sherman *et al.* 1978). Yeast cells were transformed by the lithium acetate method (Ito *et al.* 1983) or by electroporation (Becker and Guarente 1991).

Mutagenesis and isolation of novel, *PHO81*-dependent Pho^c mutants: The *PHO5-HIS3* reporter fusion was constructed as follows. The *PHO5* promoter was amplified using the polymerase chain reaction (PCR) from plasmid pMH313 (Han and Grunstein 1988) with the following two primers: 5′-GC GGAATTCGATCCGAAAGTTGCA-3′ and 5′-TTAAGCTCTA ATGGTTACCCTAGGGCG-3′. The amplified product contains nucleotides −550 to −1 of the *PHO5* promoter, which includes the Pho4p- and Pho2p-binding sites, as well as sites for three of four positioned nucleosomes that undergo transitions in low-phosphate medium (Almer and Horz 1986). This PCR fragment was cut with *Eco*RI and *Bam*HI and subcloned into pRS314 (Sikorski and Hieter 1989) to create pRS314-*PHO5*. The *HIS3* open reading frame and 3′-untranslated region, a total of 1100 bp, were amplified using PCR from plasmid pJJ217 (Jones and Prakash 1990) with the primers 5′-GCGGGATCCACAGAGCAGAAAGCC-3′ and 5′-TCCCCGCG GATCACCACAACTAAC-3′. This amplified product was cut with *Bam*HI and *Sac*II and cloned into pRS314-*PHO5* to create plasmid p*PHO5-HIS3*. Finally, nucleotides −460 to −110 of the 5′-untranslated region of *HIS3* were amplified from plasmid pJJ217 by PCR with the following primers: 5′-GCGGGGCC CCTGCACGGTCCTGTT-3′ and 5′-GCGGAATTCGAGTCAT CCGCTAGG-3′. This amplified product was cut with *Apa*I and *Eco*RI and cloned into plasmid p*PHO5-HIS3* to create plasmid p*PHO5-HIS3int*. To integrate this fusion construct into the yeast genome, plasmid p*PHO5-HIS3int* was cut with *Apa*I and *Sac*II, and the liberated 1.9-kb piece was gel purified and transformed into a *pho80*Δ strain (EY0486). The net result of the targeted integration at the *HIS3* locus was to delete most of the *HIS3* promoter and replace it with 525 bp of the *PHO5* promoter. After selection for stable transformants on SD -His, individual colonies were tested for the integration of the construct at the *HIS3* locus by colony PCR using primers 5′-TTAAGCTCTAATGGTTACCCTAGGGCG-3′ and 5′-TTTAG CTTCTCGACGTGGGCCTTT-3′. A 950-bp product was produced from cells that had undergone the integration event, but not from cells that contained random insertions. The resulting strain was crossed to a wild-type strain (EY0091). The heterozygous diploid was sporulated to generate the starting strain for the selection, EY0100.

A logarithmically growing culture of strain EY0100 in YPD was washed and resuspended in distilled water, mutagenized with UV to 33% survival, and plated onto SD -His with the addition of 2 mm 3-amino-1,2,4-triazole. These plates were incubated for 4 days at 25° in the dark. Colonies were picked and patched onto YPD plates. The Pho^c mutants were identified by assaying the histidine prototrophs for constitutive *PHO5* expression using acid phosphatase plate assays (Toh-e *et al.* 1973) on rich medium. Recessive mutants were isolated by mating the Pho^c mutants to a wild-type strain (EY0183) and assaying the resulting diploid strains for constitutive *PHO5* expression. Mutations in genes that were previously known to be required for *PHO5* repression (*i.e.*, *PHO80*, *PHO85*, or *PHO84*) were identified by crossing the recessive mutants to a *pho80*Δ *pho85*Δ double mutant (EY0179) or a *pho84*Δ mutant (EY0211) and analyzing the resulting diploids for the Pho^c phenotype using acid phosphatase plate assays. Complementation tests among the remaining non-*pho80*, non-*pho85*, and non-*pho84* recessive Pho^c mutants were performed by mating them to each other after backcrossing. *PHO5* expression was examined in these diploids by acid phosphatase plate assays.

To test whether these novel recessive mutants are *PHO81* dependent, we crossed one mutant from each group to EY0150 (*pho81*Δ*::TRP1*). The resulting diploid cells were sporulated, and the double-mutant haploid cells were identified from the nonparental ditype tetrads by examining *PHO5* expression in low-phosphate medium.

Plasmid cloning: Each novel recessive *PHO81*-dependent Pho^c mutant was backcrossed at least three times to a wild-type strain. The Pho^c phenotype segregated 2:2 for each class, suggesting that the mutation resides in a single gene. The wild-type versions of the genes containing the novel *PHO81*-dependent Pho^c mutations were cloned by screening a centromere-based (YCp50) yeast genomic library (a gift from A. Murray and K. Hardwick) for plasmids that were able to complement the Pho^c mutant phenotype in acid phosphatase plate assays. Because we found that the lithium acetate transformation method was somewhat mutagenic and that our *PHO81*-dependent Pho^c mutants picked up suppressors or reverted at a fairly high rate, we transformed the DNA library primarily by electroporation. Transformants were streaked to isolate single colonies, which were patched and tested for plasmid dependence on 5-FOA plates. Plasmids were then isolated from these transformants and retransformed into the original mutant strains to test complementation.

Phosphate uptake assays: Phosphate uptake assays were performed with a modification of a previously described procedure (Tamai *et al.* 1985). Phosphate uptake was measured on

whole cells. Yeast strains grown to log phase in synthetic high-phosphate medium were inoculated into no-phosphate medium at an optical density at 600 nm (OD_{600}) of 0.1–0.2 and grown for 4–6 hr at 30°. Cells were washed and resuspended in uptake medium at pH 4.5 or pH 3.0 to a final OD_{600} of ~0.5–0.8, and they were incubated at 30° for 20 min before uptake experiments were performed. The final external inorganic phosphate concentration was adjusted to 10 μm, and an appropriate amount of $H_3{}^{32}PO_4$ (Dupont NEN Research Products) was added as a radioactive label. The labeled substrate was added to prewarmed cells, and samples were withdrawn at different time intervals. Samples were filtered immediately through 0.8-μm nitrocellulose membrane filters (Millipore, Bedford, MA) and washed twice with 5 ml of 100 mm KH_2PO_4. The amount of phosphate taken up by the yeast cells was determined by scintillation counting.

Quantitative studies of *PHO5-GFP* expression: Log-phase yeast strains harboring p*PHO5-GFP* (EB0180) were inoculated into SD -Ura or SD low-phosphate -Ura medium. The p*PHO5-GFP* plasmid was made by fusing the *PHO5* promoter (nucleotides −550 to −1 upstream of the ATG) in-frame to the N terminus of green fluorescent protein (GFP; Chalfie *et al.* 1994) cloned into the *URA3*-marked vector pRS316 (Sikorski and Hieter 1989). Emission at 510 nm was measured after excitation at 395 nm of whole cells using a fluorometer (Photon Technology International, Inc., Monmouth Junction, NJ). The final emission was normalized to the cell density as measured by OD_{600}. Background fluorescence was determined by using wild-type cells transformed with pRS316.

Pho4-GFP localization: pACPHO4-GFP (EB0347) consists of the *PHO4* promoter (nucleotides −323 to −1 upstream of the ATG) and the entire *PHO4* coding region fused in frame to the N terminus of a GFP mutant (S65T; Heim *et al.* 1995) cloned into the *URA3*-marked vector pRS316. pACPHO4-GFP was used to transform different yeast strains. Using a BX60 microscope (Olympus Corp., Lake Success, NY), localization of the fusion protein was examined by direct fluorescence of live yeast grown in SD -Ura.

RESULTS

A genetic selection to isolate mutants that express *PHO5* constitutively: To identify novel factors that signal *PHO5* repression, we designed a selection to isolate mutants that express *PHO5* constitutively (Figure 1). Our starting strain for this selection was *his3::PHO5-HIS3* (EY0100), in which the promoter of the *HIS3* gene was replaced by the *PHO5* promoter (see materials and methods). We demonstrated that *HIS3* gene expression is subject to the same regulatory control as the *PHO5* gene in three ways. First, in high-phosphate medium, this strain was a histidine auxotroph, whereas in low-phosphate medium, it was a histidine prototroph. Second, in high-phosphate medium, a *his3::PHO5-HIS3 pho80*Δ strain was a histidine prototroph. Third, a *his3::PHO5-HIS3 pho2*Δ strain was a histidine auxotroph (data not shown). Thus, the ability to grow in the absence of histidine reflects *PHO5* expression in the strain *his3::PHO5-HIS3*.

To carry out the selection for Pho^c mutants, we plated approximately one million live, mutagenized yeast cells on SD -His plates containing aminotriazole. A total of 420 colonies of heterogenous sizes grew, and 167 of

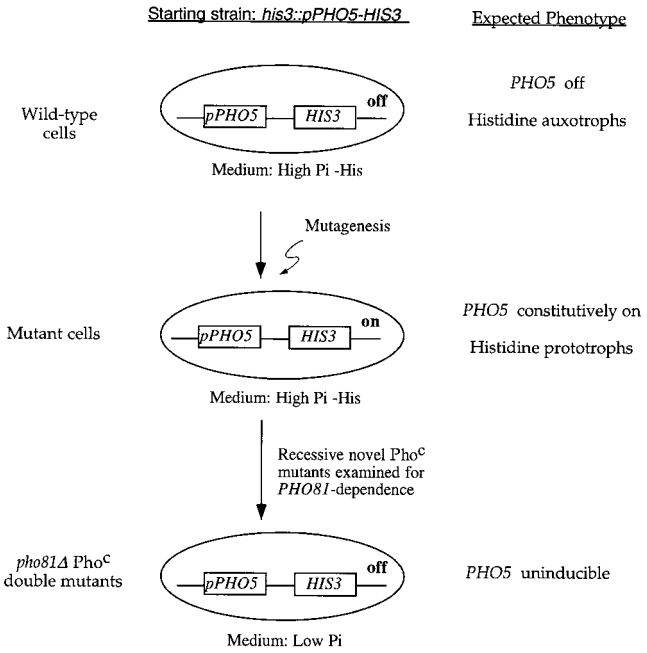

Figure 1.—Selection strategy for novel *PHO81*-dependent Pho^c mutants. The starting strain for the selection was *his3::pPHO5-HIS3* (EY0100), in which the promoter of the *HIS3* gene was replaced by the *PHO5* promoter; it is a histidine auxotroph in high-phosphate media. The mutant screen included two parts. In the first step, histidine prototrophs were selected on phosphate-rich media lacking histidine. In the second step, these mutants were screened for *PHO81*-dependence.

420 conferred unambiguously the constitutive *PHO5* expression phenotype. The Pho^c phenotype was scored by the acid phosphatase assay in which a chromagenic phosphatase substrate, α-naphthylphosphate, was applied in an agar overlay; yeast strains expressing *PHO5* turn red by these assays (Toh-e *et al.* 1973). We determined that 81 mutants contained dominant Pho^c mutations. Among the remaining 86 recessive mutants, we recovered 24 alleles of either *PHO80* or *PHO85*, 44 alleles of *PHO84*, and 18 alleles of mutations in genes not previously identified in the screen of Ueda *et al.* (1975). These 18 novel Pho^c mutants fell into eight complementation groups. Mutants in four of these complementation groups also had a slow growth phenotype at 16°. Genetic analysis has revealed that the mutation that causes the cold-sensitive (cs) phenotype is tightly linked to the Pho^c mutation in each case.

Four novel recessive Pho^c mutant groups are *PHO81* dependent: To distinguish genes involved in the signaling process from those that affect other aspects of *PHO5* regulation (*e.g.*, transcriptional repression), we analyzed the epistatic relationship between *pho81* and the novel Pho^c mutant classes. We reasoned that a *pho81* mutant should be epistatic to mutants defective in the signaling process (see also discussion); in these mutant strains, deletion of the *PHO81* gene should result in Pho4p

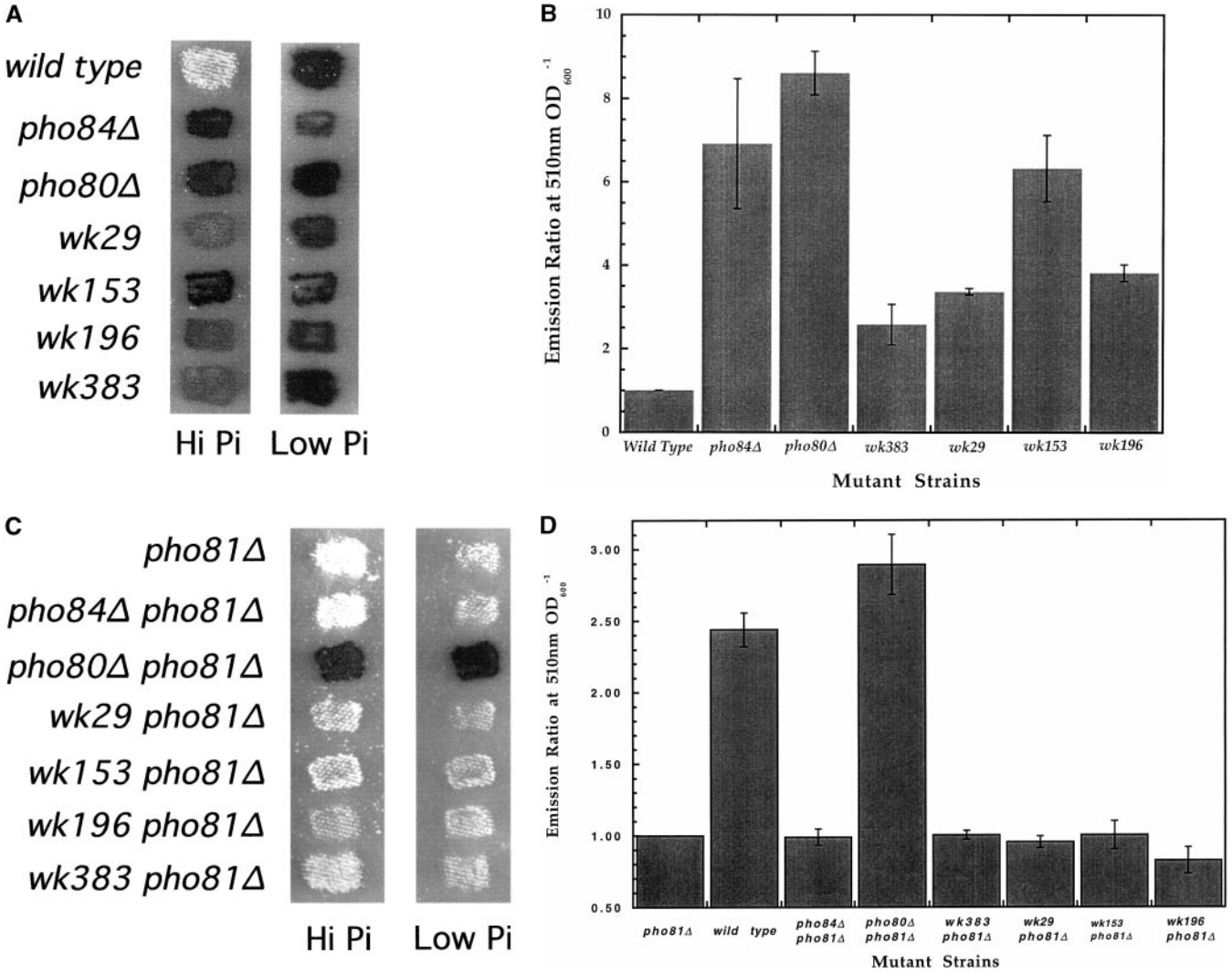

Figure 2.—Analysis of *PHO5* expression in novel, *PHO81*-dependent Pho^c mutants in the presence (A and B) and absence (C and D) of *PHO81*. (A and C) Endogenous *PHO5* expression was determined by acid phosphatase plate assays in high- (left column) and low- (right column) phosphate synthetic media. (B and D) Quantitation of the expression of *PHO5* in high- and low-phosphate media, respectively. Expression of GFP driven by the *PHO5* promoter in various strains was determined by GFP emission at 510 nm. Data are reported as the mean values of three independent experiments, and the error bars indicate the standard deviation.

being localized to the cytoplasm and an uninducible *PHO5* expression phenotype. Double mutants of *pho81* and novel Pho^c mutants were generated (see materials and methods) and examined for *PHO5* expression and Pho4-GFP localization. Four complementation groups showed a *PHO81* dependence for *PHO5* expression and Pho4-GFP localization. Each of the four groups contains only one mutant allele, namely *wk29*, *wk153*, *wk196*, and *wk383*. Mutants *wk29*, *wk153*, and *wk196* also have a slow growth phenotype at 16°.

We measured *PHO5* expression in these four *PHO81*-dependent mutants in high-phosphate medium in two ways. We first used acid phosphatase plate assays (Toh *et al.* 1973) and the color intensity of different Pho^c mutant strains as indicators of the level of *PHO5* activity (Figure 2, A and C). Then, to better quantitate *PHO5* expression, we measured *PHO5* promoter activity by using a p*PHO5-GFP* construct in which the *PHO5* promoter was fused to the GFP (EB0180; Figure 2, B and D). The strength of the *PHO5* promoter was measured by GFP fluorescence at 510 nm (see materials and methods).

Results from these two methodologies show strong correlation; mutants with a stronger Pho^c phenotype (darker color) have a higher fluorescent emission at 510 nm. Figure 2, A and B, demonstrates that the four novel *PHO81*-dependent mutants express *PHO5* constitutively, but to different degrees. Among the four *PHO81*-dependent complementation groups harboring p*PHO5-GFP*, *wk153*, which confers the strongest Pho^c phenotype, emits a GFP fluorescence at 510 nm that is approximately five- to eightfold above that of a wild-

type strain. *wk196* and *wk29* are of an intermediate Pho^c phenotype and emit GFP fluorescence approximately threefold above wild type, whereas *wk383*, which confers the weakest Pho^c phenotype, emits GFP fluorescence approximately twofold above wild type (Figure 2B).

To examine *PHO5* expression in double mutants of *pho81*Δ and the novel recessive Pho^c mutants, we performed acid phosphatase plate assays and GFP fluorescence assays in low-phosphate medium, as shown in Figure 2, C and D, respectively. Only the results for the *PHO81*-dependent mutants are shown. A *pho80*Δ *pho81*Δ double-mutant strain was included as a control for *PHO5* expression in low-phosphate medium because *PHO80* is a gene known to function downstream of *PHO81*. Similarly, a *pho84*Δ *pho81*Δ double-mutant strain was also included as a control because *pho81* is epistatic to *pho84*. The four mutant groups show a phenotype similar to that of *pho84*Δ *pho81*Δ, indicating that *pho81* is epistatic to these novel Pho^c mutants, and that they are likely to define genes that function upstream of *PHO81*. The four complementation groups whose Pho^c phenotype is independent of *PHO81* were not analyzed further in this study.

We next used the localization of Pho4-GFP in high-phosphate medium to monitor the *PHO81* dependence of our Pho^c mutants. The localization of Pho4p has been characterized previously (O'Neill *et al.* 1996). In both wild-type and *pho81*Δ strains, Pho4-GFP is localized to the cytoplasm in high-phosphate medium (Figure 3). In mutants that express *PHO5* constitutively, such as *pho84*Δ, Pho4-GFP is localized exclusively to the nucleus. However, in the double mutant *pho84*Δ *pho81*Δ, Pho4-GFP is again localized to the cytoplasm because *pho81* is epistatic to *pho84*. In contrast, in a *pho80*Δ *pho81*Δ strain, Pho4-GFP is localized to the nucleus because *pho80* is epistatic to *pho81*. Thus, the epistatic relationship between the Pho^c mutants and *pho81* can be studied easily using this assay. In the mutant *wk153*, Pho4-GFP is localized exclusively to the nucleus, while in *wk29*, Pho4-GFP is localized both to the nucleus and to the cytoplasm. It should be noted that this result correlates with the observation that *wk153* expresses more GFP when harboring the p*PHO5-GFP* construct and has higher acid phosphatase activity in high-phosphate medium than *wk29*; this is consistent with the model in which the concentration of nuclear Pho4p modulates the level of *PHO5* expression. In contrast, in the double mutants *wk153 pho81*Δ and *wk29 pho81*Δ, Pho4-GFP is predominantly cytoplasmic, indicating that *pho81* is epistatic to *wk153* and *wk29*. Thus, the Pho4-GFP localization is consistent with both the results from acid phosphatase plate assays for *PHO5* expression and the *PHO5-GFP* fluorescence assays, and it confirms that *pho81* is epistatic to *wk153* and *wk29*. The Pho4-GFP localization analysis in *wk196* and *wk383* is very similar to that of *wk29* (data not shown).

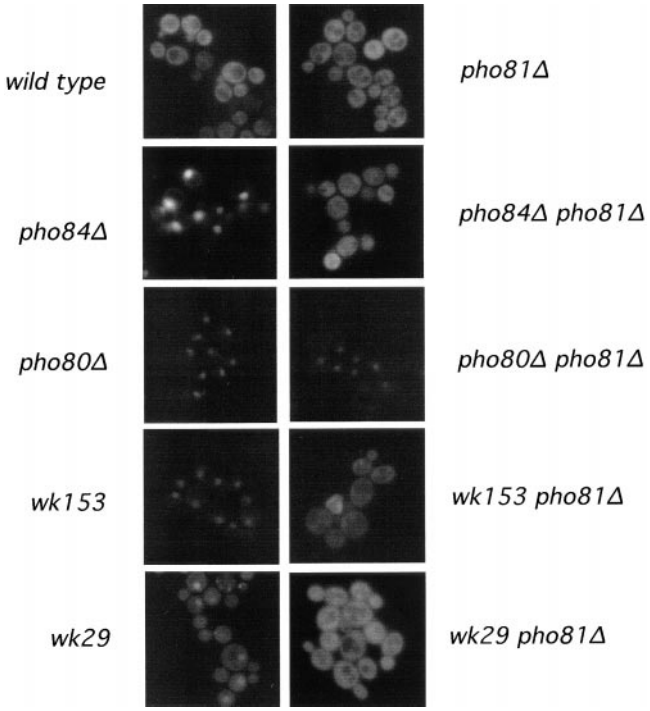

Figure 3.—Studies of the subcellular localization of Pho4-GFP in mutant strains. Direct fluorescence was used to determine the localization of Pho4-GFP in live yeast cells grown in phosphate-rich media. *wk29* and *wk153* were renamed *acc1-29* and *pho86-153*, respectively.

The novel *PHO81*-dependent Pho^c mutations represent alleles of *PHO86*, *PMA1*, *ACC1*, and *PHO23*: During our mutant characterization, Yompakdee *et al.* (1996) reported the isolation of a novel mutant that expresses *PHO5* constitutively, *pho86-1*, on the basis of a screen for arsenate-resistant mutants. Pho86p is a membrane protein with two putative transmembrane domains (Yompakdee *et al.* 1996) and has no homology to other proteins. On the basis of the following observations, we conclude that *wk153* is an allele of *PHO86*. First, *wk153* failed to complement the Pho^c phenotype of a *pho86*Δ*::TRP1* strain (EY0404). Second, after the diploid resulting from the cross of *wk153* and EY0404 was sporulated, the mutation that caused the 16° slow growth phenotype segregated away from Trp$^+$ spores, indicating that this mutation in *wk153* was tightly linked to the *PHO86* locus. Third, a low-copy-plasmid expressing *PHO86* (a gift from Nobuo Ogawa) was able to complement both the *PHO5* constitutive phenotype and the 16° slow growth phenotype of *wk153*. We therefore referred to *wk153* as *pho86-153*.

To clone the remaining *PHO81*-dependent genes, we determined which genes could complement the *PHO5* constitutive expression phenotype of each mutant by screening a low-copy yeast genomic library (see materials and methods). Both the Pho^c and the slow growth phenotype at 16° of the *wk196* mutant were complemented by two plasmids. The overlapping region of the insert fragments in these two plasmids contains two

intact ORFs, *PMA1* and *YGL007w*. Subsequent subcloning localized the complementing region to a *Hin*dIII-*Xho*I genomic fragment (EB0658), that contains only one complete ORF, *PMA1*, which encodes a p-type plasma membrane ATPase (Serrano *et al.* 1986).

To confirm that the mutation responsible for the Pho^c phenotype in *wk196* was tightly linked to the *PMA1* locus, we subcloned the *Pst*I / *Xho*I fragment of the *PMA1* ORF into a *LEU2*-marked integrating vector, pRS305 (Sikorski and Hieter 1989). This plasmid (EB0660) was linearized with *Hin*dIII, a unique site within the *PMA1* open reading frame, and was targeted to the genomic site via homologous recombination in a wild-type strain (EY0183). The integration of this vector at the *PMA1* locus was confirmed by Southern blotting (data not shown). The resulting strain (EY0469) was crossed to a *wk196* strain, and the diploid was sporulated. All 31 tetrads examined were of the parental ditype (*i.e.*, the mutation that caused the Pho^c phenotype segregated away from the Leu$^+$ spores), indicating that this mutation in *wk196* was tightly linked to the *PMA1* locus. On the basis of our complementation and linkage analyses, *wk196* was named *pma1-196*.

One plasmid (EB0657) complementing the Pho^c phenotype of the *wk29* mutant strain was isolated, and it contained only one intact ORF, *ACC1*, which encodes acetyl-CoA carboxylase. This plasmid also rescues the slow-growth phenotype of *wk29* at 16°. To demonstrate that *ACC1* corresponded to the *wk29* locus, a *Hin*dIII fragment that contained part of the *ACC1* gene was subcloned into pRS306, a *URA3*-marked integrating vector. The plasmid (EB0661) was linearized with *Nar*I and targeted to the genomic site by homologous recombination. Southern blotting confirmed the integration of the vector at the *ACC1* locus (data not shown). The resulting strain (EY0468) was crossed to the *wk29* strain, and the diploid was sporulated. All 28 tetrads examined were of the parental ditype, demonstrating linkage of *ACC1* to *wk29*. We then referred to *wk29* as *acc1-29*.

The mutant phenotype of *wk383* was complemented by three identical plasmids isolated from independent clones. Further subcloning localized the complementing region to a *Pst*I-*Spe*I genomic fragment, which was subcloned into the *Pst*I and *Spe*I sites of pRS314. The resulting plasmid (EB0659) contains only one ORF, *YNL097c*, which we named *PHO23*, and *wk383* was named *pho23-1*. The *wk383* mutation was shown to be linked to the *RAS2* gene that is adjacent to *YNL097c* by mating *wk383* to a *ras2*Δ*::LEU2* strain (a gift from J. Whistler and J. Rine) and analyzing the resulting tetrads.

To confirm that the *YNL097c* ORF corresponds to the *wk383* locus, we constructed a disruption vector. An *Eco*RI-*Sph*I fragment containing the *HIS3* gene (from pJJ215, EB0098) was inserted into the *Nco*I-*Sph*I fragment of EB659, with the *Eco*RI and *Nco*I ends blunted by treatment with Klenow. The TATA box and approximately half of the *YNL097c* ORF, including the ATG start codon, were deleted in the resulting plasmid (EB0662). EB0662 was then cut with *Eco*RI and *Not*I and transformed into a diploid wild-type cell so that one of the endogenous *YNL097c* copies in the yeast genome was replaced by one-step gene replacement (Rothstein 1983). His$^+$ transformants were selected and sporulated. All His$^+$ haploid cells also exhibited a Pho^c phenotype in >20 tetrads analyzed. The disruption of *YNL097c* was confirmed by Southern blotting (data not shown). The resulting strain has no growth defect on either YPD or synthetic media with high or low phosphate at a variety of temperatures, indicating that *PHO23* is nonessential for cell viability. The Pho^c phenotype of the *pho23*Δ*::HIS3* strain is similar to that of *pho23-1*.

PHO23 encodes a putative 330-amino-acid polypeptide, and the hydropathy profile of this protein suggests that it has no transmembrane domains. It has weak homology with two hypothetical yeast proteins, Yhr090p and Ymr075p, according to a search of the yeast genome database using the BLAST program (Altschul *et al.* 1990). Moreover, Pho23p is similar to a human protein p33ING1, a tumor suppressor functioning in the p53-signaling pathway (Garkavtsev *et al.* 1998). Interestingly, Pho23p shares significant similarity with p33ING1 at the C terminus, with 61% identity over 52 residues using the BLAST program.

Enhanced Pho^c phenotype in double mutants between *acc1*, *pma1*, and *pho23*: To test whether the *ACC1*, *PMA1*, and *PHO23* genes function in a common pathway in *PHO5* regulation, we examined the constitutive *PHO5* expression phenotype in the double mutants *acc1-29 pma1-196* (EY0464), *acc1-29 pho23*Δ (EY0465), and *pma1-196 pho23*Δ (EY0466). We examined the expression of *PHO5* in high-phosphate medium using acid phosphatase plate assays. Each of the double mutants displayed a stronger Pho^c phenotype than either parent strain (data not shown). Normally, the synergy in phenotypes in a double mutant resulting from two total loss-of-function mutations implies that the two genes do not function in a linear pathway. However, in our case, the interpretation is complicated because *ACC1* and *PMA1* are essential genes, and we isolated only partial loss-of-function alleles of these two genes. Thus, the enhanced Pho^c phenotype in these double mutants is either because these novel Pho^c genes do not function in a linear pathway signaling *PHO5* repression, or because *acc1-29* or *pma1-196* are only partial loss-of-function alleles.

Phosphate uptake analysis defines two classes of mutants: Because *PHO84* encodes a high-affinity phosphate transporter and *PHO86* is implicated to function in the phosphate transport system (Yompakdee *et al.* 1996), we performed phosphate uptake assays to test whether the remaining genes that we identified are also involved in the high-affinity phosphate transport system. Figure 4 documents the results of phosphate uptake experiments. The *pho84*Δ, *pho86*Δ, and *pma1* strains have a clear defect in phosphate uptake. This result is consis-

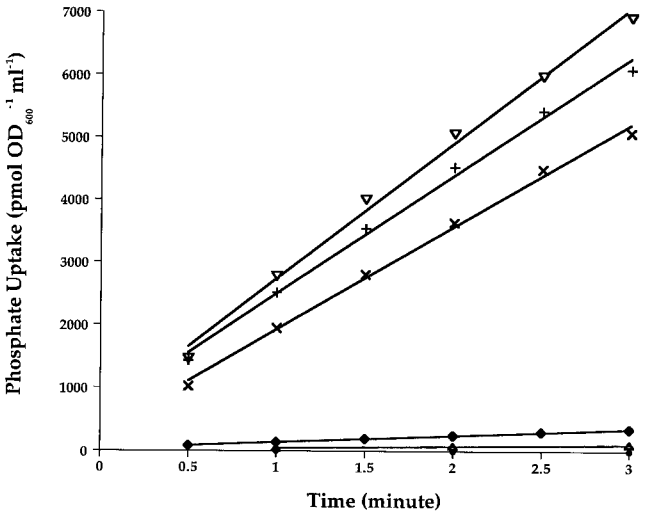

Figure 4.—Analysis of phosphate uptake in different mutants. Phosphate uptake was measured as described in materials and methods. Uptake rates (nmol min^{-1} ml^{-1} $OD_{600}{}^{-1}$) were determined at a phosphate concentration of 10 μm. ▽, EY0183 (wild-type strain), 2.17 ± 0.073; +, EY0452 (*acc1-29*), 1.88 ± 0.044; ◆, EY0454 (*pma1-196*), 0.11 ± 0.008; ×, EY0455 (*pho23-1*), 1.64 ± 0.016; Δ, EY0453 (*pho86-153*), 0.03 ± 0.05; ●, EY0211 (*pho84*Δ), not distinguishable from background noise.

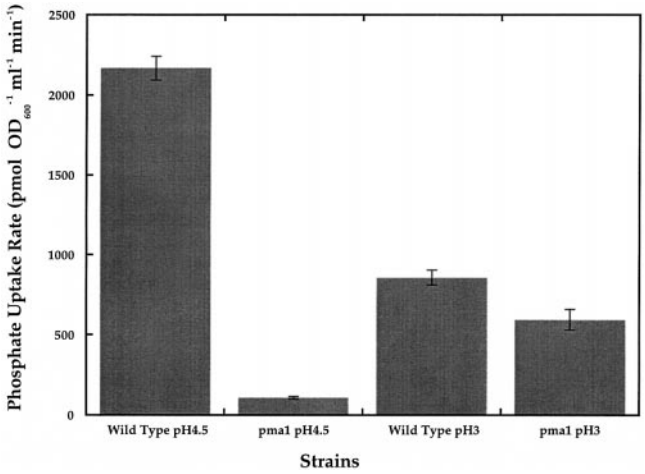

Figure 5.—The phosphate uptake defect of *pma1*-196 is rescued by lowering the pH. Phosphate uptake was measured as described in materials and methods. Uptake rates were determined at a phosphate concentration of 10 μm. Data are reported as mean values of three independent experiments, and the error bars represent the standard deviation.

tent with the function of Pma1p as a proton pump, as well as the fact that pH is able to affect phosphate uptake kinetics in yeast (Nieuwenhuis and Borst-Pauwels 1984; Tamai *et al.* 1985). In contrast, *acc1-29* and *pho23-1* are able to take up phosphate as well as a wild-type strain. Moreover, the *acc1-29 pho23*Δ (EY0465) double mutant does not have a phosphate uptake defect (data not shown), confirming that neither *ACC1* nor *PHO23* are involved in high-affinity phosphate transport.

The phosphate uptake defect of *pma1-196* can be rescued by lowering the pH: To determine which aspects of Pma1p function are required for constitutive *PHO5* expression, we obtained several *pma1* mutant strains from other labs and assayed them for *PHO5* expression. The strains we obtained have mutations in residues required for ATPase activity [A608T (van Dyck *et al.* 1990), H285Q (Wach *et al.* 1996), and A135V (Na *et al.* 1993)]; for membrane targeting [P434A and G789S (Chang and Fink 1995)], and for proton pumping activity [I183A (Wang *et al.* 1996) and S368F (Perlin *et al.* 1989)]. Only one of the mutants expressed *PHO5* constitutively; I183A conferred a weak Pho^c phenotype. This mutant has decreased proton pumping ability, although its ATPase activity is comparable to that of the wild type (Wang *et al.* 1996), raising the possibility that an impaired proton pump causes poor phosphate uptake, which in turn results in constitutive *PHO5* expression by an unknown mechanism.

To determine if the proton pumping defect in *pma1-196* is indeed the reason for poor phosphate uptake, we examined if the phosphate uptake defect of *pma1-196* could be rescued by providing a more acidic extracellular environment to restore the proton gradient across the plasma membrane. We measured phosphate uptake in a wild-type strain and *pma1-196* at pH 4.5 and pH 3.0, as shown in Figure 5. At pH 3.0, *pma1-196* could take up phosphate at about the same rate as a wild-type strain, whereas at pH 4.5, it had a phosphate uptake defect (Figures 4 and 5). We noted that the wild-type strain could take up phosphate at a higher rate at pH 4.5 than at pH 3.0, consistent with the observation that the optimum pH for the high-affinity phosphate transport system is between 4 and 5 (Nieuwenhuis and Borst-Pauwels 1984; Tamai *et al.* 1985). We were not able to investigate whether lowering the pH would rescue the Pho^c phenotype in *pma1-196* because *pma1-196* would not grow in low pH medium. These data suggest that the proton pumping defect in *pma1-196* is the reason for its poor uptake of phosphate and the resulting Pho^c phenotype.

The *PHO5* constitutive expression phenotype of *acc1-29* can be rescued by supplementation of fatty acids: The Pho^c phenotype of *acc1* indicates a possible connection between fatty acid synthesis and *PHO5* regulation. Recently, several *acc1* alleles that confer novel phenotypes have been isolated, leading to the proposal that Acc1p performs a function in addition to its role as an acetyl-CoA carboxylase (Schneiter and Kohlwein 1997). To determine if the effect of the *acc1* mutation on *PHO5* regulation was allele specific, we examined *PHO5* expression in various *acc1* mutants in high-phosphate medium, including *acc1-2150* [a temperature-sensitive fatty acid auxotroph (Mishina *et al.* 1980)], *acc1-7-1* [defective in mRNA export (Schneiter *et al.* 1996)], and *acc1cs* [synthetic lethal with *hpr1* (Guerra and Klein 1995)].

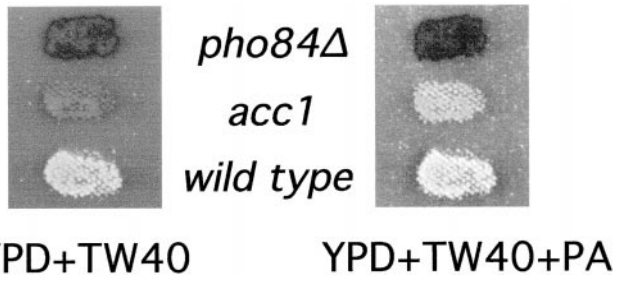

Figure 6.—The *PHO5* constitutive phenotype of *acc1-29* can be rescued by supplementation with palmitic acid (PA). *PHO5* expression was determined by acid phosphatase plate assays in the absence (left column) and presence (right column) of PA.

Constitutive *PHO5* expression was observed in all *acc1* alleles tested using acid phosphatase plate assays, indicating that the Pho^c phenotype is not allele specific, but that it can be attributed to a general defect in *acc1* mutants. To investigate if this general defect is caused by the inefficient synthesis of long chain fatty acids, we supplemented the medium with palmitic acid and examined *PHO5* expression in *acc1-29*. The Pho^c phenotype of *acc1-29* is rescued by the supplementation of palmitic acid (Figure 6), suggesting that inefficient fatty acid synthesis causes constitutive *PHO5* expression in *acc1* mutants.

DISCUSSION

Isolation of mutants defective in signaling *PHO5* repression: In this study, we have described a genetic selection for mutants that express *PHO5* constitutively in high-phosphate medium. Because we are interested in how the high-phosphate signal is generated and transduced, we have focused on mutants whose phenotype is dependent on the function of the CDK inhibitor Pho81p. Repression of the phosphate response pathway by a high concentration of extracellular phosphate is a complex process, consisting of successful detection and generation of the phosphate signal, proper activation of the kinase activity of the Pho80p-Pho85p cyclin-CDK complex, appropriate localization of Pho4p to the cytoplasm, and correct establishment of transcriptional repression (*e.g.*, repressive chromatin structure). Many mutations could interfere with these processes and cause inappropriate expression of *PHO5* in high-phosphate medium. Molecules that are involved in transducing a high-phosphate signal should be specifically required to prevent inhibition of the Pho80p-Pho85p complex by Pho81p; the *pho81* mutant will be epistatic to mutations in these signaling molecules. We isolated five mutant classes whose phenotypes are *PHO81* dependent.

Two of the five mutant classes were previously known to confer a Pho^c phenotype: *PHO84* encodes a high-affinity phosphate transporter, and *PHO86* is required *in vivo* for high-affinity phosphate uptake (Yompakdee *et al.* 1996). It is unclear if *PHO86* is a positive regulator of *PHO84* or if it is directly involved in signal transduction.

We isolated additional mutations in genes previously unknown to be involved in *PHO5* regulation. *PMA1* encodes a plasma membrane ATPase, which is required for the *in vivo* function of several transporters, including the high-affinity phosphate transport system (Vallejo and Serrano 1989; Kotyk 1994). *PMA1* is likely to be a positive regulator of *PHO84* because Pma1p functions as a proton pump and Pho84p is a proton-phosphate symporter (Borst-Pauwels 1981).

Two other classes of *PHO81*-dependent Pho^c mutants include *ACC1* and a new gene, *PHO23*. Mutations in these two genes do not cause high-affinity phosphate uptake defects, suggesting that they cause the Pho^c phenotype by mechanisms distinct from those of *PHO86* and *PMA1*. *ACC1* encodes an acetyl-CoA carboxylase, which catalyzes the rate-limiting step in the *de novo* synthesis of fatty acids. Mutant alleles of *ACC1* were initially isolated as fatty acid auxotrophs. The *ACC1* gene is essential for cell viability and is evolutionarily conserved. Its role in the phosphate metabolism pathway is unclear (see next section). Null alleles of *PHO23* result in only a partial Pho^c phenotype, which argues against Pho23p being a direct inhibitor of Pho81p function because hyperactive alleles of *PHO81* confer a much more severe Pho^c phenotype (data not shown). Alternatively, Pho23p may play a role in inhibiting Pho81p if there exist additional genes, possibly the two homologues, with redundant function. In summary, these genes are likely to affect different aspects of the transduction of the phosphate signal.

Constitutive *PHO5* expression in *acc1* mutants: In our screen for constitutive *PHO5* expression mutants, we isolated an allele of *ACC1*. We have shown that the Pho^c phenotype is not allele specific. Moreover, the Pho^c defect can be rescued by supplementing exogenous palmitic acid (Figure 6). These results suggest that the defect in *PHO5* repression in an *acc1* mutant is caused by the inefficient synthesis of fatty acids, and that there may exist crosstalk between fatty acid biosynthesis and phosphate metabolism pathways. What is the mechanism of action of *ACC1* in the signal transduction pathway for *PHO5* repression? If *ACC1* functions upstream of *PHO81*, it is possible that some metabolite(s) of fatty acids may serve as a second messenger to signal *PHO5* repression. In *acc1* mutants, this metabolite would be absent, resulting in constitutive expression of *PHO5*. Another possibility is that a protein involved in *PHO5* regulation requires specific fatty acylation for its *in vivo* function. The intracellular level of the required fatty acid in *acc1* mutants would be so low that fatty acylation is impaired, leading to the constitutive expression of *PHO5*.

Recently, some *acc1* mutants that have defects in nuclear morphology have been isolated (Schneiter and Kohlwein 1997), leading to the speculation that *ACC1* is directly involved in maintaining membrane structure. *acc1-7-1* was isolated as a mutant defective in mRNA export, and further analysis revealed a defect in the nuclear envelope of the mutant yeast strain (Schneiter *et al.* 1996). Whether the membrane structure of organelles in *acc1-29* is defective has not been investigated. The fact that the high-affinity phosphate transport system is intact in the *acc1-29* mutant suggests that there is no general defect in plasma membrane structure in this mutant strain.

Is the high-affinity transport system directly involved in signaling? Because *PHO84*, *PHO86*, and *PMA1* are required for *PHO5* repression, one intriguing possibility is that the high-affinity phosphate transport system is directly involved in signaling repression of *PHO5* transcription. There are two models that could explain the constitutive *PHO5* expression in *pho84* or *pho86* mutants. It is possible that in addition to their phosphate transport functions, Pho84p and Pho86p may also act as sensor proteins for the levels of extracellular phosphate and transduce a repression signal when phosphate levels are high. Alternatively, the phosphate transport function of the high-affinity phosphate uptake system may be only indirectly involved in the signaling process. In *pho84* or *pho86* mutants, defects in phosphate uptake might result in a low level of intracellular phosphate (or some metabolite) that serves as a messenger; a lack of this messenger may cause a defect in the production of a repression signal.

The first model, in which the phosphate transporter is also the phosphate sensor, has a precedent in bacteria. The Pho regulon of *E. coli* is also regulated by extracellular phosphate levels; its transcription is repressed when phosphate levels are high and is induced when phosphate levels are low (Wanner 1996). In the *E. coli* system, the phosphate signal is transduced by a two-component regulator complex consisting of *phoR* and *phoB*. Repression of the Pho regulon also requires an intact Pst phosphate transport system and a protein called PhoU. PhoR senses phosphate starvation signals and activates phoB, which is a transcription factor required for transcription of the Pho regulon (Wanner 1996). Mutations in the Pst transport complex that separate the transport and repression functions have been isolated, suggesting that the phosphate transporter is able to directly sense changes in the extracellular concentration of phosphate (Cox *et al.* 1988).

In yeast, there are also examples of transporters serving as sensors. It has been proposed that some glucose transporters act as receptors for sensing glucose levels. Dominant gain-of-function mutations in two yeast genes, *SNF3* and *RGT2*, which encode glucose transporters, have been isolated (Ozcan *et al.* 1996). These mutants cause induction of the expression of the glucose-regulated gene *HXT2* in the absence of glucose, suggesting that the glucose signal is transmitted into the cell by glucose transporters that also act as glucose receptors that sense extracellular glucose levels (Ozcan *et al.* 1996). Interestingly, both Snf3p and Rgt2p belong to the same family of 12-transmembrane domain transporters as Pho84p (Bun-Ya *et al.* 1991). Recently, an ammonium permease, Mep2p, was also proposed to be involved in a signal transduction pathway that regulates pseudohyphal growth in response to ammonium starvation (Lorenz and Heitman 1998).

There are also examples of intracellular metabolites that serve as signals to control gene expression. For example, intracellular concentrations of glutamine are able to regulate gene expression. In yeast, the expression of *GLN1*, which encodes a glutamine synthetase, is inactivated by an increase in the intracellular glutamine concentration (Magasanik 1991). Similarly, the expression of *glnA*, the structural gene for bacterial glutamine synthetase, is also inactivated by high levels of intracellular glutamine. This process depends on the regulation of another two-component complex with NR_{II} as the sensor and NR_I as the effector (Parkinson 1993).

Previous studies on the yeast phosphate transport system suggest that in addition to the high-affinity uptake system, there exists a constitutive low-affinity phosphate transport system with a proposed K_m of 1 mm (Nieuwenhuis and Borst-Pauwels 1984; Tamai *et al.* 1985). We supplied the medium with external phosphate $\leq$100 mm to saturate the low-affinity transport system, and we examined *PHO5* expression in *pho84*Δ and *pho86*Δ strains. The Pho^c phenotype of *pho84*Δ and *pho86*Δ strains cannot be suppressed at phosphate levels 100-fold above the K_m of the low-affinity phosphate uptake system, suggesting that these mutants may be defective in transducing a repression signal downstream rather than simply in phosphate uptake. However, whether the high-affinity phosphate uptake system can directly sense changes in extracellular phosphate levels remains to be determined.

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