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A Systematic High-Throughput Screen of a Yeast Deletion Collection for Mutants Defective in *PHO5* Regulation

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ABSTRACT

In response to phosphate limitation, *Saccharomyces cerevisiae* induces transcription of a set of genes important for survival. One of these genes is *PHO5*, which encodes a secreted acid phosphatase. A phosphate-responsive signal transduction pathway (the *PHO* pathway) mediates this response through three central components: a cyclin-dependent kinase (CDK), Pho85; a cyclin, Pho80; and a CDK inhibitor (CKI), Pho81. While signaling downstream of the Pho81/Pho80/Pho85 complex to *PHO5* expression has been well characterized, little is known about factors acting upstream of these components. To identify missing factors involved in the *PHO* pathway, we carried out a high-throughput, quantitative enzymatic screen of a yeast deletion collection, searching for novel mutants defective in expression of *PHO5*. As a result of this study, we have identified at least nine genes that were previously not known to regulate *PHO5* expression. The functional diversity of these genes suggests that the *PHO* pathway is networked with other important cellular signaling pathways. Among these genes, *ADK1* and *ADO1*, encoding an adenylate kinase and an adenosine kinase, respectively, negatively regulate *PHO5* expression and appear to function upstream of *PHO81*.

THE ability to respond appropriately to environmental changes is essential for cell survival. Microorganisms respond to nutrient limitation by regulating the expression of genes important for survival. Inorganic phosphate is an essential nutrient for the synthesis of many cellular components such as nucleic acids, phospholipids, and phospho-metabolites. The budding yeast *Saccharomyces cerevisiae* responds to changes in extracellular inorganic phosphate concentration by regulating the phosphate-responsive signaling pathway (the *PHO* pathway) (Lenburg and O'Shea 1996). As a result of *PHO* pathway signaling, many *PHO* genes are repressed under high-phosphate conditions and induced under no-phosphate conditions. One of these genes is *PHO5*, which encodes a secreted acid phosphatase.

The *PHO* pathway was originally described 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). *PHO80*, *PHO85*, and *PHO84* are required for the repression of *PHO5*, and loss-of-function mutations in these genes result in constitutive expression of *PHO5*, even under high-phosphate conditions (Ueda *et al.* 1975).

Significant progress has been made in understanding the molecular mechanism of the *PHO* signaling pathway. Central to the *PHO* pathway is a cyclin/cyclin-dependent kinase (CDK) complex, Pho80/Pho85 (Toh-e and Shimauchi 1986; Uesono *et al.* 1987; Madden *et al.* 1988; Toh-e *et al.* 1988; Kaffman *et al.* 1994), whose activity is regulated in response to external phosphate concentrations. Pho81 (Coche *et al.* 1990; Schneider *et al.* 1994; Ogawa *et al.* 1995), a CDK inhibitor, binds to Pho80/Pho85 when cells are grown under both high- and no-phosphate conditions. However, it appears that Pho81 inhibits the kinase only during phosphate starvation (Schneider *et al.* 1994). The inhibition is mediated by a novel CDK inhibitor motif (Huang *et al.* 2001). The Pho81/Pho80/Pho85 complex regulates the activity of Pho4 (Kaffman *et al.* 1994; Komeili and O'Shea 1999), a transcription factor required for *PHO5* expression. When yeast cells are grown in high-phosphate medium, Pho4 is phosphorylated by Pho80/Pho85. Phosphorylated Pho4 is localized predominantly to the cytoplasm and *PHO5* transcription is repressed (O'Neill *et al.* 1996). When yeast cells are grown in medium devoid of phosphate, the kinase activity of Pho80/Pho85 is inhibited by Pho81. Thus, Pho4 is unphosphorylated and localized to the nucleus, where it activates *PHO5* transcription.

Even though the molecular mechanism of signaling from the Pho81/Pho80/Pho85 complex to *PHO5* expression has been well characterized, the phosphate sensor is still not known, and the signaling process between the sensor and the kinase complex is not understood. *PHO84*, encoding a phosphate-starvation-inducible high-affinity

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H^+/PO_4 symporter, is suggested to function upstream of *PHO81*, because loss-of-function mutations in *PHO81* are epistatic to mutations in *PHO84* (Bun-Ya *et al.* 1991). Cells of the *pho84*Δ strain express *PHO5* constitutively, suggesting that Pho84 might be the phosphate sensor. However, overexpression of unrelated phosphate transporters or a glycerophosphoinositol transporter in the *pho84*Δ strain suppresses its constitutive phenotype, suggesting that Pho84 is not required for sensing phosphate (Wykoff and O'Shea 2001). Although traditional genetic studies have been conducted to search for a phosphate sensor and other factors that signal upstream of *PHO81* in the *PHO* pathway, the results were limited by the fact that the assays were not quantitative or systematic (Ueda *et al.* 1975; Lau *et al.* 1998).

To identify missing factors in the *PHO* pathway and to better understand signal transduction for *PHO5* regulation, we developed a high-throughput, quantitative acid-phosphatase liquid assay to screen the yeast deletion collection (Winzeler *et al.* 1999), searching for novel mutants that are defective in *PHO5* regulation. We wished to identify signaling components that function upstream of *PHO81* and are required for the regulation of *PHO5*. As a result of this study, we have identified and confirmed at least nine functionally diverse genes that were previously not known to regulate *PHO5* expression. When each of these genes was deleted, the resulting yeast strains displayed either constitutive *PHO5* expression under high-phosphate conditions or reduced *PHO5* expression upon phosphate starvation. Analysis of these mutants suggests that *ADK1* and *ADO1* (Konrad 1988; Lecoq *et al.* 2001), encoding an adenylate kinase and an adenosine kinase, respectively, negatively regulate *PHO5* expression and appear to function upstream of *PHO81*.

MATERIALS AND METHODS

Yeast strains and growth conditions: All yeast deletion strains used in this study had the entire open reading frame of each gene deleted as indicated. For the initial screen and characterization, all strains except *kcs1*Δ were taken from the yeast deletion collection (*MAT***a** haploid complete set) (Winzeler *et al.* 1999). The *kcs1*Δ strain from the yeast deletion collection used for this study appeared to contain an additional mutation(s), which resulted in a constitutive phenotype similar to a *pho80*Δ strain (data not shown). *KCS1* is known to have only modest effects on Pho5 expression when it is deleted (Steger *et al.* 2003). For this work, we replaced the *kcs1*Δ strain from the yeast deletion collection with a *kcs1*Δ strain (EY1258) that exhibited the same phenotype as previously published (Steger *et al.* 2003). For the independently generated deletion strains, all genes were inactivated using a PCR-based deletion protocol that deleted the entire open reading frame (Kitada *et al.* 1995). Deletion of the appropriate genes was confirmed by PCR and, in some cases, by phenotypic analysis. For disruptions that were initially performed on diploid strains (EY519, EY1406, EY1407, EY1509, EY1510, and EY1600; Table 1), the resulting heterozygous diploids were sporulated, and the tetrads were dissected to isolate haploids with the desired gene deletions (Guthrie and Fink 1991). Yeast strains used in this study are listed in Table 1. All strains were grown in standard yeast medium as described (Guthrie and Fink 1991). No-phosphate medium was prepared as described (Lau *et al.* 1998).

High-throughput liquid acid-phosphatase assay: Yeast cultures were incubated at 30° in a HiGro incubator shaker (Gene Machines), centrifugations were performed at 3000 rpm in a Beckman GS-6KR centrifuge (Beckman, Fullerton, CA), high-throughput liquid assays were conducted in a 96-well format at room temperature using a Biomek FX 96-channel pipetting robot (Beckman), and the OD_{600} or OD_{420} was measured in a Spectra Max 340 plate reader (Molecular Devices, Menlo Park, CA). Each 96-well plate of the yeast deletion collection (Winzeler *et al.* 1999) was thawed and inoculated onto a YEPD plate using a 96-pin tool and then incubated for 2 days. The strains were then inoculated into a 96-well plate containing 600 μl of SD complete medium in each well using a 96-pin tool and grown overnight to saturation. Cells grown to saturation and cells grown at midlog phase showed similar Pho5 activity. Each culture was spun down and washed two times with 600 μl of no-phosphate medium and resuspended in 200 μl of no-phosphate medium. Fifty microliters of cell suspension was then reinoculated into 850 μl of no-phosphate medium and incubated at 30°. Every 120 min after transfer to no-phosphate medium, 200 μl of each culture was withdrawn and the OD_{600} was measured. To start the assay, 50 μl of each culture was added to 200 μl of *p*-nitrophenylphosphate (5.62 mg/ml in 0.1 M sodium acetate, pH 4.2), mixed, and incubated at room temperature for 15 min. The reaction was stopped by the addition of 200 μl of ice-cold 10% trichloroacetic acid. A total of 200 μl of the reaction mixture was then withdrawn, added to 200 μl of saturated sodium carbonate solution, mixed, and spun for 10 min at 3000 rpm. Finally, 200 μl of the supernatant was removed and the OD_{420} was measured. The units of phosphate activity were expressed as $OD_{420}/OD_{600} \times 1000$. The mutant candidates identified from the initial screen were reorganized into a new set of 96-well plates and a secondary liquid assay was performed in the same manner.

***PHO5* mRNA analysis:** Yeast strains were grown to an OD_{600} of 0.4–1.0 in SD complete medium in 96-well plates as for the high-throughput liquid acid-phosphatase assay described above. A 1-ml culture of each strain was then transferred into a microcentrifuge tube, and total RNA was extracted by standard acid phenol treatment (Guthrie and Fink 1991), reverse transcribed using Stratascript reverse transcriptase following the manufacturer's instructions (Stratagene, La Jolla, CA), and treated with RNaseA. As a control, each sample was additionally mock treated (without the reverse transcriptase). For each reverse transcription (RT) reaction, a *PHO5* RT primer (5′-TTG TCTCAATAGACTGGCGTTGTAA) and an *ACT1* RT primer (5′-TGGTGAACGATAGATGGACCA) were added into the same reaction. Appropriately diluted RT products were then analyzed by quantitative PCR in real time using an Opticon continuous fluorescence detection system (MJ Research, Watertown, MA) and primers amplifying *PHO5* (nucleotides 630–760) or *ACT1* (nucleotides 715–816). For each strain, the relative level of *PHO5* mRNA was normalized to that of *ACT1*. For most of the samples, the mock signals were <1% of the actual signals.

Fluorescence microscopy: All microscopy experiments were performed as described (Huang *et al.* 2001).

RESULTS

Isolation of mutants defective in Pho5 induction in response to phosphate starvation from a systematic screen of the yeast deletion collection: We developed a systematic high-throughput quantitative acid-phosphatase liq-

TABLE 1

Strains of *S. cerevisiae* used in this study

Strain	Genotype	Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (S288C)	Brachmann (1998)
BY4742	*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* (S288C)	Brachmann (1998)
YKO	BY4741 Yeast KO collection (*KAN1*) *MAT***a** set	Winzeler (1999)
K699	*MAT***a** *ade2-1 trp-1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL*$^+$	Nasmyth (1990)
EY519	K699 *MAT*α *pho81Δ::HIS3*	This work
EY1258	BY4741 *kcs1Δ::HIS3*	This work
EY1406	K699 *ado1Δ::LEU2*	This work
EY1407	K699 *MAT*α *ado1Δ::LEU2*	This work
EY1509	K699 *MAT*α *pho81Δ::HIS3 ado1Δ::LEU2*	This work
EY1510	BY4741 *pho81Δ::KAN1 adk1Δ::LEU2*	This work
EY1580	BY4741 *PHO4-YFP::KAN1*	This work
EY1591	BY4741 *PHO4-YFP::KAN1 pho85Δ::HIS3*	This work
EY1592	BY4741 *PHO4-YFP::KAN1 pho80Δ::HIS3*	This work
EY1593	BY4741 *PHO4-YFP::KAN1 reg1Δ::HIS3*	This work
EY1594	BY4741 *PHO4-YFP::KAN1 adk1Δ::HIS3*	This work
EY1595	BY4741 *PHO4-YFP::KAN1 ado1Δ::HIS3*	This work
EY1596	BY4741 *PHO4-YFP::KAN1 ykl169cΔ::HIS3*	This work
EY1597	BY4741 *PHO4-YFP::KAN1 mot2Δ::HIS3*	This work
EY1598	BY4741 *PHO4-YFP::KAN1 pho81Δ::HIS3*	This work
EY1599	BY4741 *PHO4-YFP::KAN1 pho4Δ::HIS3*	This work
EY1600	BY4741 *PHO4-YFP::KAN1 snf2Δ::HIS3*	This work
EY1601	BY4741 *PHO4-YFP::KAN1 pho2Δ::HIS3*	This work
EY1602	BY4741 *PHO4-YFP::KAN1 spt7Δ::HIS3*	This work
EY1603	BY4741 *PHO4-YFP::KAN1 ada3Δ::HIS3*	This work
EY1604	BY4741 *PHO4-YFP::KAN1 fur4Δ::HIS3*	This work
EY1605	BY4741 *PHO4-YFP::KAN1 gcn5Δ::HIS3*	This work
EY1606	BY4741 *PHO4-YFP::KAN1 ada2Δ::HIS3*	This work
EY1607	BY4741 *PHO4-YFP::KAN1 ino4Δ::HIS3*	This work
EY1608	BY4741 *PHO4-YFP::KAN1 vps24Δ::HIS3*	This work
EY1609	BY4741 *PHO4-YFP::KAN1 swi3Δ::HIS3*	This work
EY1610	BY4741 *PHO4-YFP::KAN1 alt1Δ::HIS3*	This work
EY1611	BY4741 *PHO4-YFP::KAN1 snf6Δ::HIS3*	This work
EY1615	BY4741 *PHO4-YFP::KAN1 kcs1Δ::HIS3*	This work

uid assay (see materials and methods) to screen the yeast deletion collection and searched for novel mutants that were defective in *PHO5* regulation. The yeast deletion collection consists of 4848 *MAT***a** haploid strains, each lacking a single nonessential gene (Winzeler *et al.* 1999). In contrast to previous genetic studies (Ueda *et al.* 1975; Lau *et al.* 1998), our high-throughput liquid phosphatase assay is a highly sensitive and quantitative method to examine Pho5 expression *in vivo*. Furthermore, the gene mutated in each strain in the yeast deletion collection is known and facilitates rapid analysis. In our initial screen, cells were transferred to no-phosphate conditions, sampled, and assayed for Pho5 phosphatase activity every 120 min for 360 min. This enabled us to identify mutants with kinetic defects in *PHO5* induction. We used a kinetic assay because deletion of some genes involved in *PHO5* regulation results in kinetic defects in *PHO5* expression, but no defects in induction measured after overnight growth in no-phosphate medium (Barbaric *et al.* 2001; Steger *et al.* 2003).

During the initial screen, each 96-well plate from the yeast deletion collection was cultured and assayed for Pho5 acid-phosphatase activity upon phosphate starvation as described in the materials and methods. The data from each plate were then graphed and analyzed (Figure 1A). Upon phosphate starvation, a range of induced Pho5 expression in the population was observed. The mean (μ) and standard deviation (σ) were calculated for the entire population at each time point. To identify potential mutant candidates, we then assigned a control range ($\mu \pm \sigma$) (Figure 1). Strains that had a lower Pho5 activity than the control range at three of four time points were called uninducible mutant candidates, and strains that had a higher Pho5 activity than the control range were called hyperinducible mutant candidates. As expected, known mutants in the *PHO* pathway were located outside of this control range (*e.g.*, *pho80Δ* and *pho4Δ*) (Toh-e *et al.* 1973). A histogram of the 240-min data points of the entire library is shown in Figure 1B. The histograms of other time points have similar distribution patterns (data not shown). To define a manageable number of mutant candidates for the

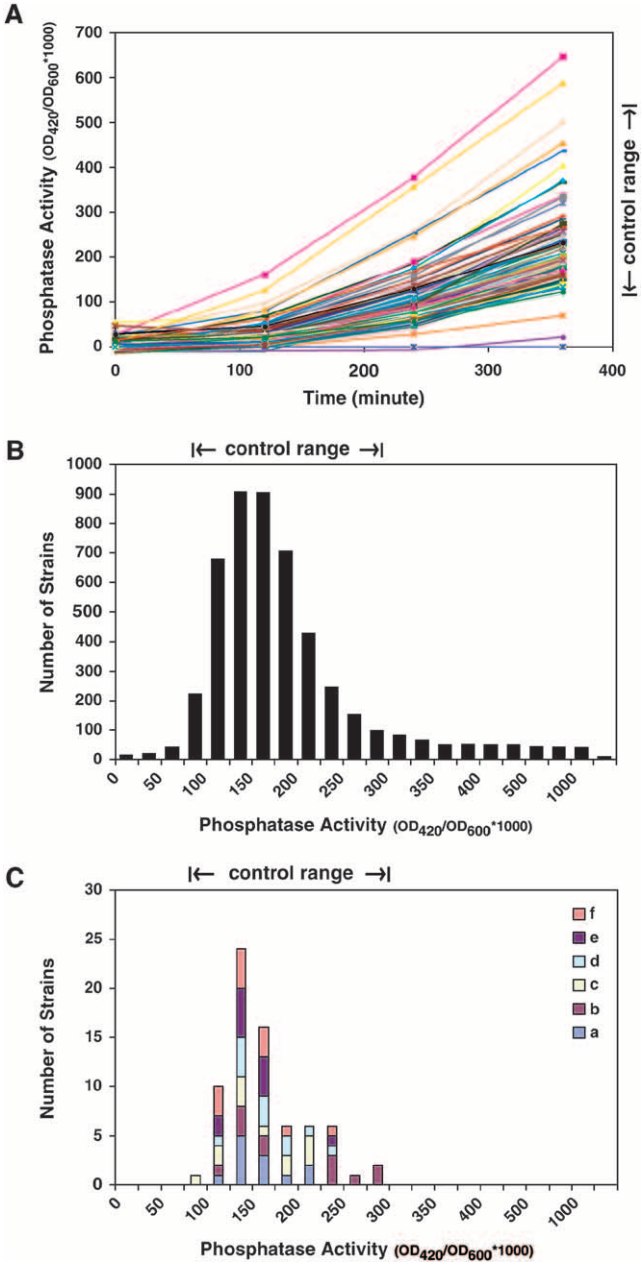

FIGURE 1.—A systematic screen of a yeast deletion collection for mutants defective in *PHO5* regulation. (A) A time course of acid-phosphatase activity in strains from a typical plate (no. 37) from the yeast deletion collection (WINZELER *et al.* 1999) in response to phosphate starvation. The majority of yeast strains exhibited a range of phosphatase activities within the control range, which covers one standard deviation (σ) above or below the mean (μ) of the entire deletion library at every time point (see RESULTS). Strains with phosphatase activity above the control range were classified as hyperinducible mutant candidates; strains with phosphatase activity below the control range were classified as uninducible mutant candidates. (B) A histogram of acid-phosphatase activity of the entire deletion library at the 240-min time point following transfer to no-phosphate medium. The majority of yeast strains exhibited phosphatase activity within the control range ($\mu \pm \sigma$). (C) A histogram of acid-phosphatase activity from six independent repeats (a–f, each in a different color) of the 12 wild-type controls (*spo75*Δ, *syn8*Δ, *yll023c*Δ, *yal068c*Δ, *fun30*Δ, *nup60*Δ, *yal065c*Δ, *tpo1*Δ, *yll029w*Δ, *yll020c*Δ, *erv46*Δ, and *cln3*Δ (SGD, http://www.yeastgenome.org) at the 240-min time point. The profiles of these 12 controls are consistently superimposed within the control range ($\mu \pm \sigma$) and closely resemble the histogram of the entire deletion library (above).

scope of this study, we selected 100 uninducible candidates with Pho5 activity that is at least one standard deviation from the mean, and 240 hyperinducible candidates with Pho5 activity that is at least two standard deviations from the mean.

To confirm the Pho5 expression phenotypes of these 340 candidates, we reorganized them into four new 96-well plates, each of which also contained 12 wild-type (WT) controls from the same library. These 12 controls were obtained by simple random sampling to cover the control range (Figure 1) and do not have a known *PHO* phenotype. In each plate, we also included an additional six known mutants that cover the spectrum of *PHO* phenotypes: *pho80*Δ, *pho85*Δ, *pho81*Δ, *pho4*Δ, *arg82*Δ, and *snf6*Δ (TOH-E *et al.* 1973; UEDA *et al.* 1975; NEEF and KLADDE 2003; STEGER *et al.* 2003). These four plates were cultured, assayed, and analyzed in the same manner as the initial screen. The profiles of the 12 wild-type controls from six independent experiments are consistently superimposed on the control range described above (Figure 1C). Of these 340 candidates, 240 showed reproducible defects in Pho5 induction: 160 were hyperinducible and 80 were uninducible. For the follow-up studies, we selected the 62 most statistically significant hyperinducible mutant candidates ($>\mu + 5\sigma$) and the 28 most statistically significant uninducible mutant candidates ($<\mu - 2\sigma$).

Initial characterization of the mutant candidates: The 90 most statistically significant mutant candidates (62 hyperinducible and 28 uninducible) were isolated because they displayed altered Pho5 induction under no-phosphate conditions as described above. To distinguish the hyperinducible candidates that have constitutive Pho5 expression (constitutive mutants) under both high- and no-phosphate conditions from candidates that have hyperinducible Pho5 expression only under no-phosphate conditions, we cultured and assayed the 62 hyperinducible candidates for Pho5 expression in high-phosphate medium at the 300-min time point. The average of three independent measurements is shown in Figure 2A. Of the 62 hyperinducible candidates, 48 exhibited significantly elevated Pho5 activity under high-phosphate conditions as compared to the average value of the 12 wild-type controls ($>\mu + 2\sigma$). Since these 48 candidates exhibited significantly constitutive Pho5 expression in high-phosphate medium, we referred to them as "constitutive mutant candidates."

The 28 uninducible mutant candidates ($<\mu - 2\sigma$) were also cultured in no-phosphate media, assayed, and analyzed in the same manner as in the initial screen. The average of three independent measurements at a 360-

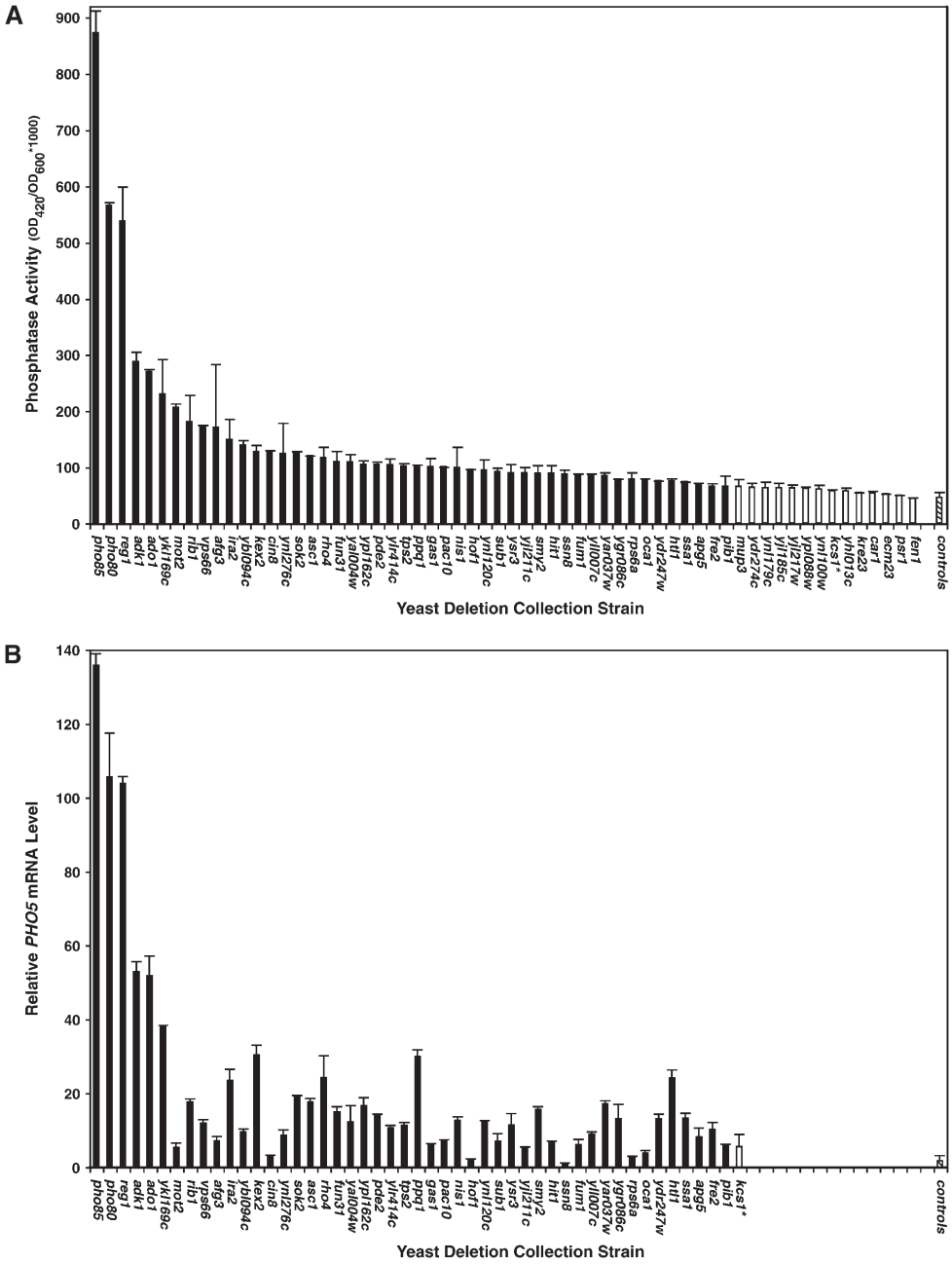

FIGURE 2.—Initial characterization of the constitutive mutant candidates. (A) Phosphatase activity of the 62 hyperinducible mutant candidates grown in high-phosphate medium. Forty-eight candidates (constitutive mutants, solid columns) exhibited significantly elevated Pho5 activity compared to the average of the 12 wild-type controls (hatched column on the far right; $>\mu + 2\sigma$). The remaining 14 candidates exhibited less elevated Pho5 activity (open columns; $<\mu + 2\sigma$). All yeast strains were from the yeast deletion collection used for the screen, except the *kcs1* deletion strain (EY1258, Table 1), which was generated independently (see MATERIALS AND METHODS). (B) *PHO5* mRNA levels in the 48 constitutive mutants grown in high-phosphate medium. For each strain, total RNA was isolated and analyzed by RT-QPCR. The level of *PHO5* mRNA was normalized to *ACT1* mRNA in the same sample. The yeast strains are listed in the same order as in A.

min time point is shown in Figure 3A. As expected, these 28 uninducible candidates showed significantly reduced Pho5 activity as compared to the controls ($<\mu - 2\sigma$).

***PHO5* mRNA analysis of the strongest mutant candidates:** To distinguish the mutants that affect Pho5 regulation at the level of transcription from the mutants that affect other processes, we performed *PHO5* mRNA analysis on the most statistically significant mutant candidates (48 hyperinducible and 28 uninducible).

If a constitutive mutant has defects in *PHO5* transcription, the *PHO5* mRNA level should be elevated compared to the wild-type strain. If a constitutive mutant instead has defects only in Pho5 protein production or secretion, the *PHO5* mRNA level will be similar to the wild-type strain. To differentiate between these types of mutants, we quantitated *PHO5* mRNA levels (normalized to *ACT1*) in the 48 constitutive mutants under high-phosphate conditions at the 300-min time point using reverse transcription-quantitative polymerase chain reaction (RT-QPCR) (MATERIALS AND METHODS). We found that the relative *PHO5* mRNA levels in 44 of the 48 constitutive mutant candidates are elevated at least twofold over the average of the 12 wild-type controls (Figure 2B). The *ssn8*Δ, *hof1*Δ, *rps6A*Δ, and *cin8*Δ strains exhibited no significant differences from the controls, suggesting that they might affect Pho5 regulation through effects on process(es) other than transcription. The *pho85*Δ, *pho80*Δ, and *reg1*Δ strains from the yeast deletion collection have

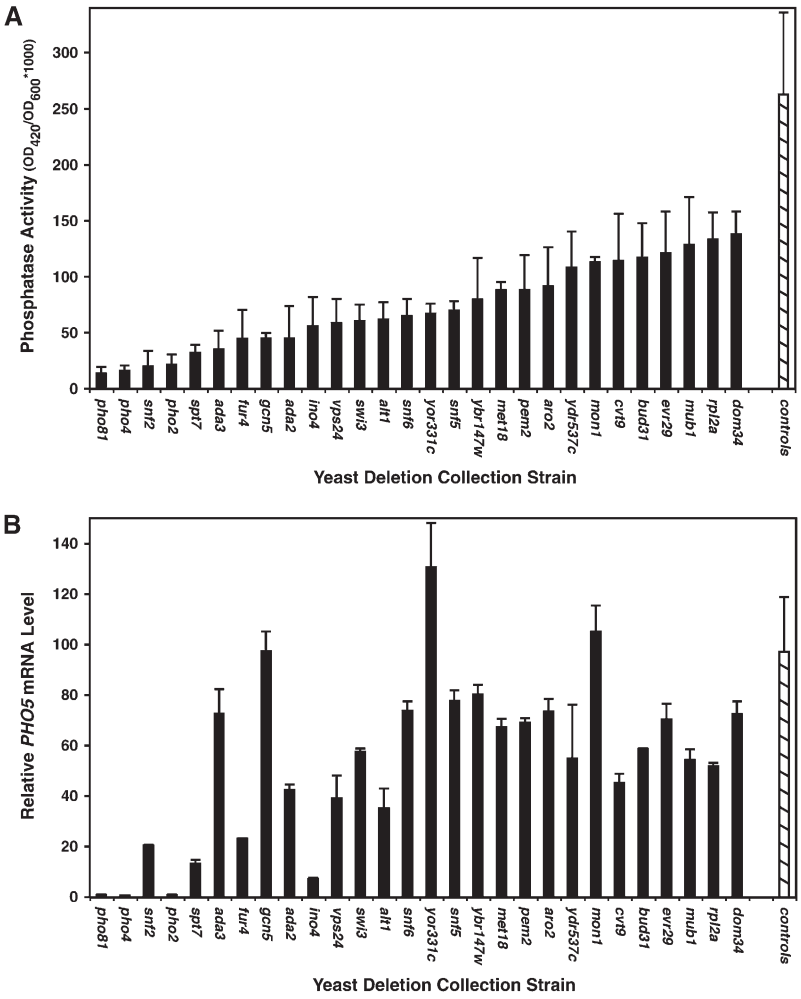

FIGURE 3.—Initial characterization of the uninducible mutant candidates. (A) Phosphatase activity of the 28 uninducible mutant candidates grown in no-phosphate medium at the 360-min time point. All 28 uninducible candidates exhibited significantly reduced Pho5 activity as compared to the average of the 12 wild-type controls (hatched column on the far right; $<\mu - 2\sigma$). All yeast strains were from the yeast deletion collection used for the screen. (B) *PHO5* mRNA levels in the 28 uninducible mutants grown in no-phosphate media. For each strain, total RNA was isolated and analyzed by RT-QPCR. The level of *PHO5* mRNA was normalized to *ACT1* mRNA in the same sample. The yeast strains are listed in the same order as in A.

the most elevated levels of *PHO5* mRNA (>60-fold more than the controls). Among the rest of mutants, the *adk1*Δ and *ado1*Δ strains have the most elevated levels of *PHO5* mRNA (>30-fold more than the controls).

Similarly, if an uninducible mutant has defects in *PHO5* transcription, the *PHO5* mRNA level should be reduced compared to the wild-type strain under no-phosphate conditions. The *PHO5* mRNA levels of the 28 uninducible candidates under no-phosphate conditions (at the 360-min time point) were analyzed in the same manner as the constitutive candidates described above. We found that the relative *PHO5* mRNA levels in 16 of the 28 uninducible mutant candidates are at least twofold less than the average in the 12 wild-type controls (Figure 3B). The *pho4*Δ, *pho81*Δ, and *pho2*Δ strains from the yeast deletion collection had the lowest levels of *PHO5* mRNA (>100-fold less than the controls). Among the rest of uninducible candidates, the *ino4*Δ, *spt7*Δ, and *snf2*Δ strains had the most significant reduction in the levels of *PHO5* mRNA (5- to 15-fold less than the controls).

For several of the uninducible and constitutive mutants we observed discrepancies between Pho5 activity levels and *PHO5* mRNA levels. As suggested above, this lack of correlation may reflect a role for these gene products in Pho5 regulation downstream of transcription. However, in some cases, the lack of correlation may result from global effects of a mutation on gene expression. For example, mutations that affect both *ACT1* and *PHO5* mRNA levels will appear as if they do not affect *PHO5* mRNA because of the way in which we have normalized the data. This is particularly a concern for some of the uninducible mutants with defects in known transcriptional components.

Confirmation of the mutant candidates: Before characterizing the mutant candidates further, it was essential to confirm that the phenotype of each candidate was the result of the deletion of the gene as indicated in the collection. It is possible that a deletion strain from the yeast deletion collection used for this study might contain additional mutation(s), which could be responsible for the phenotype observed. This was the case for the *kcs1*Δ strain from the yeast deletion collection used in our study (MATERIALS AND METHODS). For the scope of this study, we chose to independently regenerate strains corresponding to the strongest constitutive mutant candidates, which exhibited Pho5 activity at least fourfold higher than that of the controls under high-phosphate conditions (*pho85*Δ, *pho80*Δ, *reg1*Δ, *adk1*Δ, *ado1*Δ, *ykl169c*Δ,

and *mot2*Δ; Figure 2A) along with the *kcs1*Δ strain as an additional control. We also reconstructed the strongest uninducible mutant candidates that exhibited a Pho5 activity at least fourfold less than that of the controls under no-phosphate conditions (*pho81*Δ, *pho4*Δ, *snf2*Δ, *pho2*Δ, *spt7*Δ, *ada3*Δ, *fur4*Δ, *gcn5*Δ, *ada2*Δ, *ino4*Δ, *vps24*Δ, *swi3*Δ, *alt1*Δ, and *snf6*Δ; Figure 3A). These 22 deletion strains were constructed from a starting strain that has the same genetic background as the yeast deletion collection, containing *PHO4-YFP* integrated at the *PHO4* locus (EY1580; Table 1). These reconstructed deletion strains were cultured, assayed for Pho5 activity, and analyzed in the same manner as described above. If the original phenotype of each candidate were the sole result of the deletion of the gene as indicated in the deletion library, these newly constructed mutant strains should exhibit a similar *PHO* phenotype. Among the seven strongest constitutive mutant strains, five showed profiles similar to the original phenotypes (*pho85*Δ, *pho80*Δ, *adk1*Δ, *ado1*Δ, and *mot2*Δ, Figures 2A and 4A). The newly constructed *reg1*Δ strain exhibited a modest phenotype similar to *kcs1*Δ, and the new *ykl169c*Δ strain appeared to behave like the wild-type strain. Among the 14 top uninducible mutant strains, 13 showed profiles similar to the original phenotypes (*pho81*Δ, *pho4*Δ, *snf2*Δ, *pho2*Δ, *spt7*Δ, *ada3*Δ, *gcn5*Δ, *ada2*Δ, *ino4*Δ, *vps24*Δ, *swi3*Δ, *alt1*Δ, and *snf6*Δ strains; Figures 3A and 4B). The newly constructed *fur4*Δ strain appeared to behave like the wild-type strain. The time course analysis of Pho5 induction in these 14 uninducible mutants also yielded the same result (data not shown).

Pho4-YFP localization in the confirmed mutants: Among these 20 confirmed genes whose deletion affects Pho5 regulation, 9 were previously not known to be involved in regulating *PHO5* expression: *ADK1*, *ADO1*, *MOT2*, *REG1*, *ADA3 INO4 SWI3*, *VPS24*, and *ALT1* (Saccharomyces Genome Database, SGD, http://www.yeastgenome.org). To distinguish genes involved in the signaling process upstream of the Pho80/Pho85 complex from those that affect other aspects of *PHO5* regulation (*e.g.*, transcriptional repression), we monitored localization of a Pho4-YFP fusion protein. Pho4 localization in different mutant backgrounds reflects the activity of Pho80/Pho85 and indicates whether the gene acts upstream or downstream of the kinase complex in the *PHO* pathway. In the *reg1*Δ, *mot2*Δ, and *kcs1*Δ constitutive mutant strains, we found that Pho4-YFP was localized to the cytoplasm under high-phosphate conditions, suggesting that *REG1*, *MOT2*, and *KCS1* may act downstream of the kinase complex (Figure 5A). In contrast, we found that Pho4-YFP was localized to the nucleus in the *ado1*Δ and *adk1*Δ constitutive mutant strains under high-phosphate conditions, suggesting that *ADK1* and *ADO1* may act upstream of the kinase complex in the *PHO* pathway (Figure 5A). In all of the uninducible mutant strains (*e.g.*, *pho2*Δ, *snf2*Δ, *ada3*Δ, and *gcn5*Δ; data not shown for the others) except *pho81*Δ, we found that Pho4-YFP was localized to the nucleus under no-phosphate conditions, suggesting that these genes may act downstream of the kinase complex (Figure 5B).

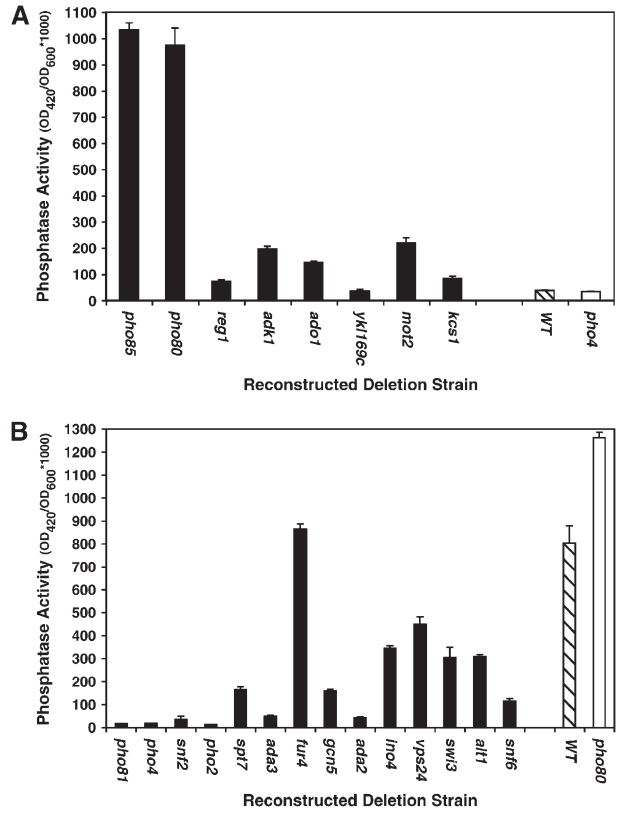

FIGURE 4.—Confirmation of the strongest mutant candidates. (A) Phosphatase activity in the seven reconstructed constitutive mutant candidates grown in high-phosphate medium. These seven constitutive candidates initially exhibited the highest levels of Pho5 activity, which was at least fourfold higher than the average of the controls (Figure 2A). *pho85*Δ, *pho80*Δ, *reg1*Δ, *adk1*Δ, *ado1*Δ, *ykl169c*Δ, and *mot2*Δ (EY1591–EY1597, respectively) were reconstructed from a wild-type (WT) strain (EY1580, Table 1) containing *PHO4-YFP* integrated at the *PHO4* locus. (B) Phosphatase activity in 14 reconstructed uninducible candidate strains grown in no-phosphate medium at the 360-min time point. These 14 uninducible candidates initially exhibited reduced Pho5 activity that was at least fourfold lower than the average of the controls (Figure 3A). *pho81*Δ, *pho4*Δ, *snf2*Δ, *pho2*Δ, *spt7*Δ, *ada3*Δ, *fur4*Δ, *gcn5*Δ, *ada2*Δ, *ino4*Δ, *vps24*Δ, *swi3*Δ, *alt1*Δ, and *snf6*Δ (EY1598–EY1611, respectively) were reconstructed from the wild-type strain (EY1580, Table 1).

***PHO81* dependence of *ADO1* and *ADK1*:** To confirm our conclusions from the Pho4-YFP localization study, we analyzed the epistatic relationship of *PHO81* to *ADO1* and *ADK1*. We reasoned that a *pho81* mutant should be epistatic to mutants defective in the signaling process upstream of the kinase complex. In these mutant strains, deletion of the *PHO81* gene should result in cytoplasmic localization of Pho4 and an uninducible Pho5 expression phenotype, whereas the mutants that affect other aspects of *PHO5* regulation (*e.g.*, transcriptional repression) will be epistatic to the *pho81*Δ mutant. Double mutants of *pho81*Δ *ado1*Δ and *pho81*Δ *adk1*Δ were generated

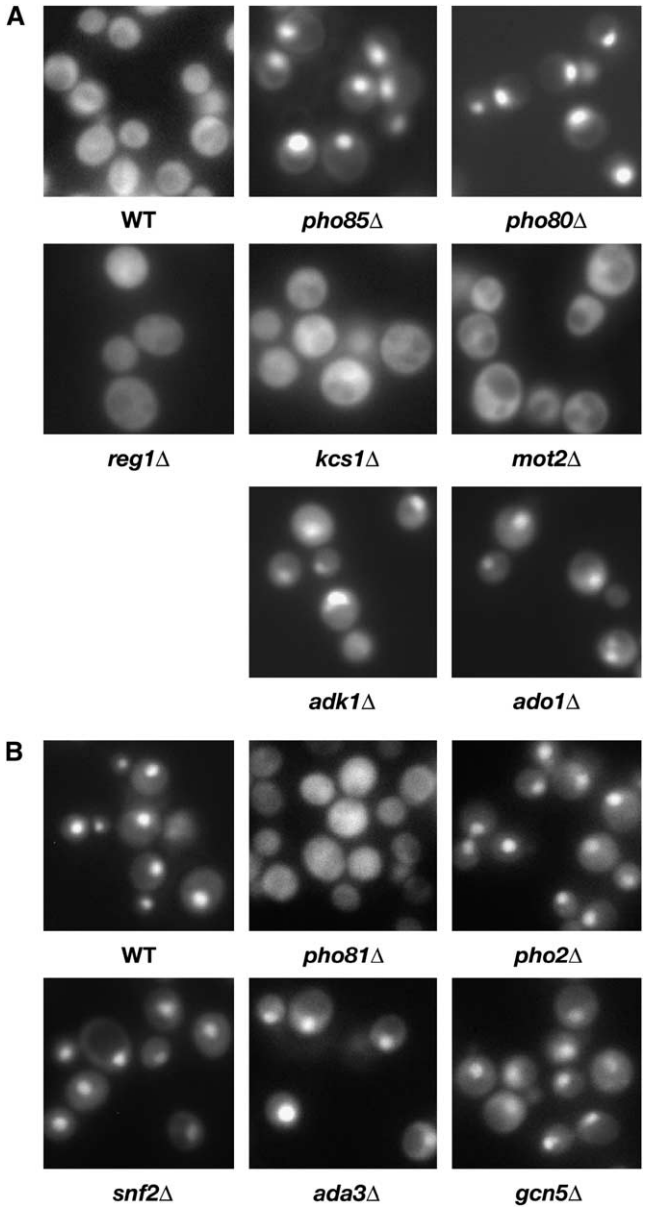

FIGURE 5.—Pho4-YFP localization studies in the strongest mutants. (A) Localization of Pho4-YFP in the strongest constitutive mutant strains grown in high-phosphate medium. The following strains were used: WT (EY1580), *pho85*Δ (EY1591), *pho80*Δ (EY1592), *reg1*Δ (EY1593), *kcs1*Δ (EY1615), *mot2*Δ (EY1597), *adk1* (EY1594), and *ado1*Δ (EY1595). (B) Localization of Pho4-YFP in the strongest uninducible mutant strains grown in no-phosphate medium. The following strains were used: WT (EY1580), *pho81*Δ (EY1598), *pho2*Δ (EY1601), *snf2*Δ (EY1600), *ada3*Δ (EY1603), and *gcn5*Δ (EY1605).

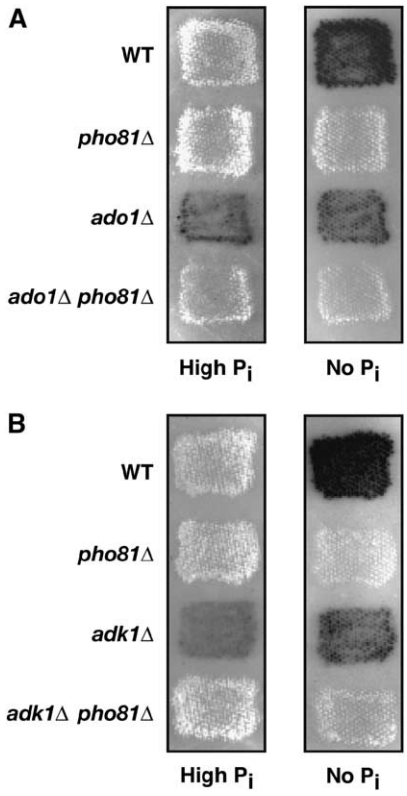

FIGURE 6.—*PHO81* dependence of *ADO1* and *ADK1*. (A) A *pho81*Δ *ado1*Δ (EY1509) double mutant was generated and examined for Pho5 expression by acid-phosphatase plate assay. A wild-type strain (K699 *MAT*α) induced Pho5 expression upon phosphate starvation (lighter color in high-phosphate medium and darker color in no-phosphate medium). The *pho81*Δ yeast strain (EY519) did not induce Pho5 expression (lighter color). In contrast, the *ado1*Δ yeast strain (EY1407) constitutively expressed Pho5 in both high- and no-phosphate media (dark color). A *pho81*Δ *ado1*Δ strain exhibited an uninducible Pho5 expression phenotype (lighter color) similar to the *pho81*Δ strain, indicating *PHO81* dependence of the *ado1*Δ phenotype. (B) A *pho81*Δ *ado1*Δ (EY1510) double mutant was generated and examined for Pho5 expression by acid-phosphatase plate assay. The wild-type strain (BY4741) induced Pho5 expression upon phosphate starvation (lighter color in high-phosphate medium and darker color in no-phosphate medium). A *pho81*Δ yeast strain (WINZELER *et al.* 1999) did not induce Pho5 expression (lighter color). In contrast, the *ado1*Δ yeast strain (WINZELER *et al.* 1999) constitutively expressed Pho5 in both high- and no-phosphate media (dark color). Similarly, *pho81*Δ *ado1*Δ exhibited an uninducible Pho5 expression phenotype (lighter color) similar to the *pho81*Δ strain, indicating *PHO81* dependence of the *adk1*Δ phenotype.

and examined for *PHO5* expression by acid-phosphatase plate assay (Figure 6). Both *ADO1* and *ADK1* showed a *PHO81* dependence for Pho5 expression. As expected, in these double-mutant backgrounds Pho4-GFP was localized to the cytoplasm (data not shown). We conclude that *ADK1* and *ADO1* act upstream of *PHO81* in the *PHO* pathway.

DISCUSSION

In an effort to better understand the signaling process in the *PHO* pathway, we have conducted a high-throughput and systematic enzymatic screen for mutants that are defective in *PHO5* regulation. We wished to identify genes that function upstream of *PHO81* and are required for *PHO5* repression. Our study identified and confirmed 20 genes that appear to be involved in *PHO5* regulation. Among these genes, 7 result in a constitu-

tive *PHO* phenotype, and 13 result an uninducible *PHO* phenotype when deleted (Figure 4). Of these 20 genes, 9 were previously not known to be involved in *PHO5* regulation (repression—*ADK1*, *ADO1*, *MOT2*, *REG1*; induction—*ADA3*, *INO4*, *SWI3*, *VPS24*, *ALT1*).

Among the constitutive mutants, the *pho80*Δ and *pho85*Δ strains showed the most elevated levels of Pho5 phosphatase activity and *PHO5* mRNA under high-phosphate conditions (Figures 2 and 4A), consistent with their central role in the *PHO* pathway. Complete loss of the kinase activity (Pho80/Pho85) results in full activation of the transcription factor Pho4, which then leads to full expression of *PHO5*. Interestingly, the *adk1*Δ and *ado1*Δ strains had the most elevated levels of Pho5 phosphatase activity and *PHO5* mRNA under high-phosphate conditions among the rest of the constitutive mutants (Figures 2 and 4A), suggesting that these two genes might play important roles in *PHO5* repression. Consistent with this hypothesis, *ado1*Δ and *adk1*Δ are the only strains that had nuclear Pho4-YFP localization similar to *pho80*Δ and *pho85*Δ strains (Figure 5A) and that exhibited phenotypes that were *PHO81* dependent, as seen in the epistatic analysis (Figure 6). These results suggest that *ADK1* and *ADO1* may act upstream of *PHO81* in the *PHO* pathway (Figure 7).

Adk1 and Ado1 play important roles in the regulation of adenosine nucleotides: *ADK1* encodes an adenylate kinase (Konrad 1988; Abele and Schulz 1995), which catalyzes the interconversion of nucleotides between AMP and ADP; *ADO1* encodes an adenosine kinase (Lecoq *et al.* 2001), which catalyzes the salvage synthesis of adenine monophosphate from adenosine and ATP. Little is known about these nucleotide kinases in yeast. Yeast cells carrying a disrupted *ADK1* locus showed a significant decrease in the level of nucleoside triphosphates (Konrad 1988). The physiological role of Ado1 is suggested to be to recycle adenosine produced by the methyl cycle (Lecoq *et al.* 2001). Why would *ADK1* and *ADO1*, which are involved in adenosine nucleotide regulation, act upon the *PHO* pathway? Since inorganic phosphate is essential for nucleotide synthesis, it is possible that nucleotide regulation is connected to the *PHO* signaling pathway to coordinate these two processes under different nutrient and growth conditions. When inorganic phosphate or nucleotide levels are high, repression of *PHO5* expression may conserve energy. Adk1 and Ado1 might repress the inhibitory activity of the CKI, Pho81, leading to activation of the kinase, Pho80/Pho85, which then inactivates the transcription factor Pho4 required for *PHO5* expression. On the other hand, if inorganic phosphate or nucleotide levels are low, repression of Pho81 by Adk1 and Ado1 might be relieved. *PHO5* expression might then be induced, generating more inorganic phosphate, which would lead to an increase of nucleotide synthesis. Consistent with this hypothesis that Adk1 and Ako1 may regulate *PHO5* expression in response to phosphate conditions, it has been shown that Ado1 activity is dependent on the presence of inorganic phosphate and other ions (Maj *et al.* 2000, 2002).

How might Adk1 and Ado1 repress Pho81? The Pho81/Pho80/Pho85 complex localizes to the nucleus where the regulation of the kinase complex is expected to take place (Kaffman *et al.* 1998; Huang *et al.* 2001). Both Adk1 and Ado1 were found in the cytoplasm and nucleus (Huh *et al.* 2003). Adk1 was also detected in a Pho85-associated complex, which included Pho81, in a high-throughput analysis of protein-protein interaction (Ho *et al.* 2002). One possible model is that Adk1 inhibits Pho81 by physical contact that is regulated by intracellular inorganic phosphate concentrations. When the concentration of intracellular inorganic phosphate is high, the physical interaction of Adk1 with Pho81 may repress Pho81's inhibitory activity. When intracellular inorganic phosphate is low, the interaction between Adk1 and Pho81 may change (*e.g.*, via a protein conformational switch), leading to the activation of Pho81. Dissecting the interactions between Adk1 and the kinase complex might help us to understand the regulation of Pho81 and how the *PHO* signaling process is connected to nucleotide regulation.

In addition to the known *PHO* genes, the four novel genes (*ADK1*, *ADO1*, *MOT2*, and *REG1*) and one known gene (*KCS1*) required for *PHO5* repression under high-phosphate conditions can be classified into four different subclasses (Figure 7, right). The first subclass plays an important role in regulation of the adenosine nucleotides: *ADO1* and *ADK1*. Our study suggests that they are the only two new genes that might act upstream of *PHO81* in the *PHO* pathway. The second subclass consists of *MOT2*, encoding a global transcriptional regulator. Mot2 has effects on the expression of many genes involved in diverse pathways (Cade and Errede 1994; Irie *et al.* 1994; Leberer *et al.* 1994; Lenssen *et al.* 2002). We observed that *PHO5* mRNA levels were elevated in the *mot2*Δ strain (Figure 2B), suggesting that the transcription of *PHO5* might be repressed by Mot2. Consistent with our findings, it has been shown that deletion of *MOT2* caused increased transcription of another *PHO* gene, *PHO84* (Irie *et al.* 1994). Our Pho4-YFP localization study suggests that *MOT2* acts downstream of the kinase complex, Pho81/Pho80/Pho85 (Figure 5A). Mot2 may act directly at the *PHO5* promoter or via a more indirect mechanism. It is also interesting to note that the deletion of *MOT2* resulted in an increase in Pho5 activity similar to the deletions of *ADO1* or *ADK1* (Figures 2A and 4A), whereas the *PHO5* mRNA level in the *mot2*Δ strain is significantly less than that in the *ado1*Δ or *adk1*Δ strains (Figure 2B). This suggests that Mot2 might regulate Pho5 expression at another level in addition to transcription. It is also possible that the *MOT2* deletion strain exhibits pleiotropic transcriptional defects that affect both *PHO5* and *ACT1*, the gene we used for normalization of mRNA levels.

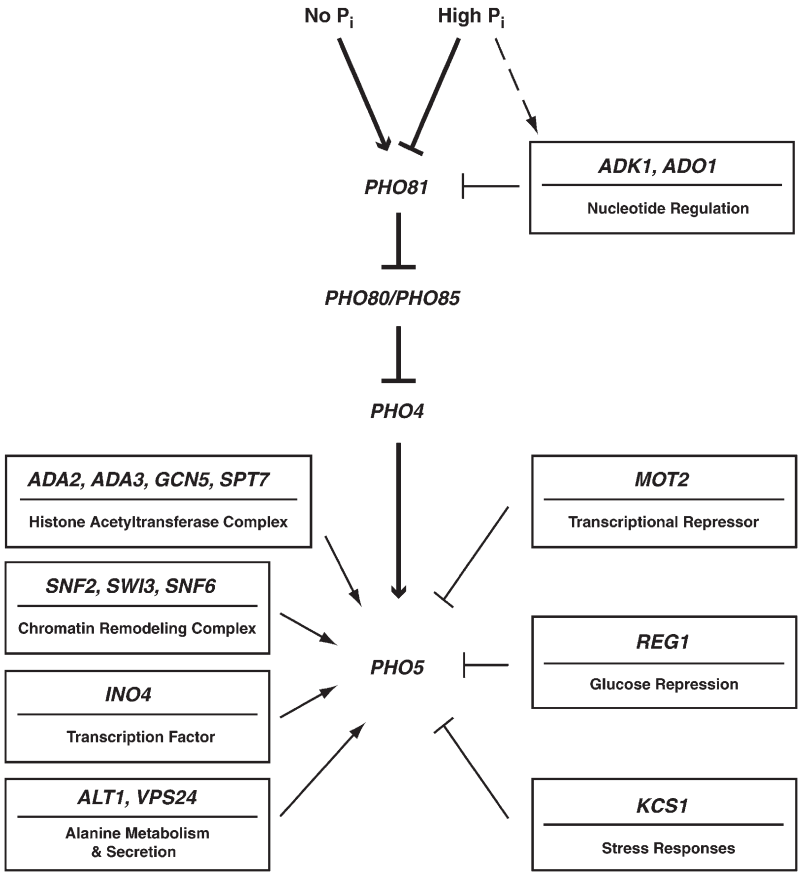

FIGURE 7.—The *PHO* pathway might be networked with other cellular signaling pathways that are important for cell survival. Genes required for efficient *PHO5* induction under no-phosphate conditions are boxed on the left next to the known *PHO* pathway, whereas genes required for *PHO5* repression under high-phosphate conditions are boxed on the right. Regulation of adenosine nucleotides might have an effect on the *PHO* pathway by acting upstream of *PHO81*, while the other processes seem to contribute to *PHO5* regulation at levels of transcription and other processes downstream of *PHO4*.

The third subclass of genes involved in the *PHO5* repression consists of genes involved in glucose repression and includes *REG1* (GANCEDO 1998), which has been shown to negatively regulate transcription of glucose-repressive genes such as *ADH2* (DOMBEK *et al.* 1999). Since the *PHO* pathway is one of the fundamental metabolic regulatory pathways in yeast, it is possible that the glucose repression pathway and the *PHO* pathway are coordinately regulated. Consistent with this hypothesis, it has been shown that phosphate and glucose cooperate to activate the protein kinase A pathway (GIOTS *et al.* 2003).

The fourth subclass of genes required for *PHO5* repression appears to be involved in the stress response: *KCS1* is required for the synthesis of inositol pyrophosphates, which are essential for vacuole biogenesis and the cell's response to certain environmental stresses (SAIARDI *et al.* 2000; DUBOIS *et al.* 2002). *KCS1* is shown to be required for repression of *PHO* genes by phosphate (EL ALAMI *et al.* 2003; STEGER *et al.* 2003). By networking the *PHO* signaling pathway and these general survival processes, yeast might benefit by a better response to environmental changes.

Among the 13 confirmed uninducible mutants, the *pho81*Δ, *pho4*Δ, and *pho2*Δ strains showed no significant *PHO5* expression upon phosphate starvation (Figures 3 and 4B), consistent with their critical roles in the *PHO* pathway. In addition to the known *PHO* genes, our study also identified five novel genes (*ADA3*, *INO4*, *SWI3*, *VPS24*, and *ALT1*) and five known genes (*ADA2*, *GCN5*, *SPT7*, *SNF2*, and *SNF6*) that are involved in Pho5 induction upon phosphate starvation. All these genes appeared to act downstream of *PHO4* since Pho4-YFP is localized to the nucleus in these deletion mutants under no-phosphate conditions (Figure 5B). These genes involved in efficient Pho5 induction can be classified into four different subclasses (Figure 7, left). The first subclass consists of genes that encode for the components of the SAGA histone acetyltransferase (HAT) chromatin-remodeling complex: *ADA2*, *ADA3*, *GCN5*, and *SPT7* (Figures 3 and 4B) (ROTH *et al.* 2001; NARLIKAR *et al.* 2002). Gcn5 and Spt7 have been shown to be required for the transcriptional regulation of *PHO5* (GREGORY *et al.* 1998; KUO *et al.* 1998; NISHIMURA *et al.* 1999; BARBARIC *et al.* 2001, 2003; NEEF and KLADDE 2003). Ada2 and Ada3 are the SAGA components that regulate the HAT activity of Gcn5. As expected, Ada2 is required for chromatin remodeling at the *PHO5* promoter by regulating Gcn5 (BARBARIC *et al.* 2003). Consistent with these findings, our study also confirmed the roles of Gcn5, Spt7, and Ada2 in *PHO5* regulation. Furthermore, we found that Ada3 was necessary for efficient *PHO5* induction (Figures 3 and 4B). Strains lacking Ada3 and Gcn5 have similar profiles in which *PHO5* mRNA expression was delayed compared to wild type (data not shown; Figure 3B) (BARBARIC *et al.* 2001), suggesting similar roles for these two SAGA components in *PHO5* regulation.

The second subclass of genes required for efficient

Pho5 induction upon phosphate starvation consists of the components of the SWI/SNF ATP-dependent chromatin-remodeling complex: *SNF2* (*SWI2*), *SWI3*, and *SNF6* (Peterson and Tamkun 1995; Smith *et al.* 2003). The SWI/SNF complex has been shown to be required for efficient remodeling of *PHO5* promoter chromatin structure: *PHO5* chromatin remodeling appears to be defective in the *snf6*Δ strain (Steger *et al.* 2003), while induction of the Pho5 acid-phosphatase activity requires Snf2 (Swi2) (Neef and Kladde 2003). Consistent with these findings, our study confirmed the roles of Snf2 and Snf6 in Pho5 regulation and also showed that Swi3 is involved in *PHO5* induction, similar to Snf6 (Figures 3 and 4B).

The third subclass of genes required for efficient Pho5 induction includes *INO4*, which encodes a transcription factor required for derepression of inositol-choline-regulated genes involved in phospholipid synthesis, such as *INO1* (Ambroziak and Henry 1994). We found that Ino4 was also required for efficient *PHO5* induction in response to phosphate starvation. Since phosphate is required for the synthesis of inositol phosphates and phospholipid synthesis, it is possible that the *PHO* pathway might be linked to these pathways. Ino4 may act directly on the *PHO5* promoter, similar to its regulation of *INO1* promoter, or via a more indirect mechanism.

The last subclass of genes required for efficient *PHO5* induction appears to be involved in alanine metabolism and protein secretion: *ALT1* encodes a putative alanine transaminase (Tatusov *et al.* 2000) and *VPS24* encodes a component for a vesicle-mediated transport system involved in protein secretion (Babst *et al.* 1998). These genes are not likely to be involved in signaling and are more likely to regulate *PHO5* expression through the steps of protein synthesis and secretion. Alternatively, they may act more indirectly to modulate Pho5 expression.

As a result of this study, we were able to identify and confirm 20 genes that appear to be involved in *PHO5* regulation. Since we chose to screen the yeast deletion collection, any essential gene involved in the *PHO5* regulation would not be identified. It is also important to note that, for the scope of this study, we characterized only the 20 genes that showed the strongest phenotype when deleted. There are still many interesting candidates with weaker phenotypes that can be validated in future studies. For example, these candidates include known genes, such as *ARG82* encoding an inositol polyphosphate kinase, which is required for chromatin remodeling of the *PHO5* promoter (Steger *et al.* 2003) and *PHO23*, encoding a probable component of the Rpd3 histone deacetylase complex, which is involved in transcriptional regulation of *PHO5* (Lau *et al.* 1998). We did not isolate 2 of the known genes that cause significant constitutive expression of *PHO5:PHO84* encoding a phosphate starvation-inducible high-affinity H^+/PO_4 symporter, and *PHO86*, encoding a protein specifically required for packaging of Pho84 (Lau *et al.* 2000). This is most likely due to the fact that *pho84*Δ and *pho86*Δ strains can quickly accumulate suppressor mutations (our unpublished results). It was reported by others that *ASF1* is required for the transcription of *PHO5* (Adkins *et al.* 2004). However, we did not observe any significant defect in *PHO5* expression (no more than 1.5-fold less than the wild type throughout an 8-hr time course) in the *asf1*Δ strain from the yeast deletion collection or in the *asf1*Δ strains that we generated in various strain backgrounds (data not shown).

In summary, this systematic high-throughput study enabled us to identify 20 genes that are involved in *PHO5* regulation. We were able not only to confirm the roles of 11 known genes in the *PHO* pathway, but also to isolate 9 novel genes previously unknown to be involved in *PHO5* regulation. Among these genes, *ADK1* and *ADO1* might act upstream of *PHO81* in the *PHO* pathway, suggesting that the *PHO* signaling process is connected to nucleotide regulation. The functional diversity of these 9 genes suggests that the *PHO* pathway might be networked with other important cellular signaling pathways that are important for cell survival (Figure 7). This might enable the yeast cells to better respond to extracellular nutrient changes and conserve energy for survival. A comprehensive analysis of the complete set of constitutive and uninducible mutants will yield important information for understanding the signaling network that allows yeast cells to respond appropriately to environmental changes.

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