The induction of yeast *Saccharomyces cerevisiae* gene *PHO5* expression is mediated by transcription factors PHO2 and PHO4. PHO4 protein has been reported to be phosphorylated and inactivated by a cyclin-CDK (cyclin-dependent kinase) complex, PHO80-PHO85. We report here that PHO2 can also be phosphorylated. A Ser-230 to Ala mutation in the consensus sequence (SPIK) recognized by cdc2/CDC28-related kinase in PHO2 protein led to complete loss of its ability to activate the transcription of *PHO5* gene. Further investigation showed that the Pro-231 to Ser mutation inactivated PHO2 protein as well, whereas the Ser-230 to Asp mutation did not affect PHO2 activity. Since the PHO2 Asp-230 mutant mimics Ser-230-phosphorylated PHO2, we postulate that only phosphorylated PHO2 protein could activate the transcription of *PHO5* gene. Two hybrid assays showed that yeast CDC28 could interact with PHO2. CDC28 immunoprecipitate derived from the YPH499 strain grown under low phosphate conditions phosphorylated GST-PHO2 in *vitro*. A phosphate switch regulates the transcriptional activation activity of PHO2, and mutations of the (SPIK) site affect the transcriptional activation activity of PHO2 and the interaction between PHO2 and PHO4. BIacore® analysis indicated that the negative charge in residue 230 of PHO2 was sufficient to help PHO2 interact with PHO4 in *vitro*.

PHO2 (also known as GRF10, BAS2) (1, 2), a protein of 559 amino acids (3), is currently known as a transcriptional activator of *PHO5* (4), *PHO81* (5), *HIS4* (2), *CYC1* (6), *TRP4* (7), *HO* (8), and *ADE1*, *ADE2*, *ADE3*, 7, and *ADE8* genes (9) in *Saccharomyces cerevisiae*. It was first identified as a positive regulatory factor that, together with a second DNA binding factor PHO4 (4), activated the transcription of *PHO5, PHO11, PHO81*, and *PHO84* in the so-called *S. cerevisiae* PHO system.

The transcription of *PHO5* gene encoding a secreted acid phosphatase in *S. cerevisiae* is regulated in response to the extracellular concentration of inorganic phosphate through a system consisting of the products of at least five genes, *PHO2, PHO4 PHO80, PHO85*, and *PHO81* (10). A current model for the acid phosphatase regulation proposes that the two positive regulatory factors encoded by *PHO4* and *PHO2* are indispensable for the transcription of *PHO5* (10). In high phosphate medium, the complex consisting of cyclin-dependent kinase PHO85 and cyclin PHO80 phosphorylates and presumably activates the transcriptional factor PHO4 (11). When the concentration of phosphate in the medium is sufficiently low, PHO81 protein, which is a cyclin-dependent kinase inhibitor, inhibits the kinase activity of the PHO80-PHO85 complex (12), thus allowing the hypophosphorylated PHO4 protein, together with PHO2 protein, to activate the transcription of *PHO5* gene.

PHO2 has four conspicuous regions (see Fig. 1). The first one is a glutamine-rich region (amino acids 23–52), the deletion of which was showed to have no effect on PHO2 function (13). The other three regions include the homeodomain (amino acids 77–136) (14), the acidic region (amino acids 294–329), and the region (amino acids 241–258) with homology to PHO80 protein. The PHO2 homeodomain is directly involved in DNA recognition. The acidic region of PHO2 contains continuous stretches of aspartic acid and asparagine. The PHO80 homologous region may be an association domain of PHO2 with PHO4, the deletion of which inactivated the PHO2 protein (13). The deletions at the C-terminal end of PHO2 protein may go up to residue 408 without strongly affecting the derepression of *PHO5* (15).

The results of the two-hybrid assay (16) argued that DNA binding by PHO4 is dependent on the phosphate-sensitive interaction with PHO2. It was also suggested that interaction with PHO2 increases the accessibility of the activation domain of PHO4 (17). Recently, immunoprecipitation experiments and protein binding assays showed that PHO2 and PHO4 form a complex with a DNA fragment and interacted with each other directly in *vitro* (18).

Protein phosphorylation has been found to play an important role in the control of diverse cellular processes, especially that of transcriptional factors in the regulation of gene expression. PHO80-PHO85 cyclin-CDK (CDK, cyclin-dependent kinase) complex can regulate the expression of *PHO5* gene by phosphorylating PHO4 (11). Cyclic AMP-dependent protein kinase (protein kinase A) has also been observed to exert its function in the synthesis of repressible acid phosphatase (19). As well, cdc2/CDC28 type kinase can affect gene expression by phosphorylation. The consensus motif of sites phosphorylated by cdc2/CDC28-type kinase is (Ser/Thr)-Pro-X-(Lys/Arg) (20). This sequence is found more frequently in nuclear proteins involved in transcriptional regulation than in proteins generally. Many of these proteins also contain DNA binding motif such as zinc fingers or helix-turn-helix structures (21–24).

In this report we describe the identification of a potential phosphorylation site near the PHO80 homologous region of PHO2 protein. The mutation of the site can affect the function of PHO2 protein. Moreover, the introduction of a negative charge to this residue seems to be necessary for PHO2 functions. We also demonstrate that PHO2 can be phosphorylated by CDC28 kinase in *vitro*. 

*This work was supported by National Natural Science Foundation of China Grant 39893320. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 86-21-64374430 (ext. 256); Fax: 86-21-64338357; E-mail: aosz@sunm.shcnc.ac.cn.
Experimental Procedures

Yeast Strains and Media—The wild type S. cerevisiae strain used in this work was YPH499 (Stratagene) with the following genotype: MATa, ura3-52, lys2-801, ade2-101, trp1-1563, his3-220, leu2-3,112, can1, gal1, gal80, URA3, GAL7, which was used in two-hybrid assay systems (24, 25) containing a HIS3 disruption of PHO4. Yeast culture were grown at 30 °C in either YPD (1% yeast extract, 2% glucose, 2% peptone) or synthetic medium (0.67% yeast nitrogen base, 2% glucose) supplemented with the appropriate amino acids as required. All yeast transformations were done using the high efficiency transformation method (26). Gene work was YPH499 (Stratagene) with the following genotype: MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-190, leu2-3,112, can1, gal1, gal80, URA3, GAL7, which was used in two-hybrid assay systems (24, 25) containing a HIS3 disruption of PHO4.

Yeast culture were grown at 30 °C in either YPD (1% yeast extract, 2% glucose, 2% peptone) or synthetic medium (0.67% yeast nitrogen base, 2% glucose) supplemented with the appropriate amino acids as required. All yeast transformations were done using the high efficiency transformation method (26). Gene work was YPH499 (Stratagene) with the following genotype: MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-190, leu2-3,112, can1, gal1, gal80, URA3, GAL7, which was used in two-hybrid assay systems (24, 25) containing a HIS3 disruption of PHO4.

Phosphorylation of PHO2

Site-directed Mutagenesis of the PHO2 Gene—Five synthetic oligonucleotides were used for site-directed mutagenesis with a U-DNA mutagenesis kit (Roche Molecular Biochemicals): 5′-ATCGATCCTGG-TATTTTTCTTT-3′, 5′-GAGAACAATACGCGCAATATAA-3′, 5′-GGTGGAG-AATACACAGTCGAATATAATA-3′, 5′-ATGCAATACAGTAATACAAGA-3′, 5′-ATCGCAATACAGTAATACAAGA-3′. A 1.0 kb PsiI/HindIII fragment of the PHO2 gene was cloned into the M13mp18 vector to produce single-stranded DNA. The mutagenesis procedures were then conducted according to the kit protocols. The mutants were isolated and sequenced to ensure that the intended base changes were present. The fragments were ligated into the M13mp18 vector containing the de- signed mutation, was then cloned back to pRS2.

Activity Assay of Acid Phosphatase—The strains to be tested were grown in 3 ml of synthetic medium lacking Leu overnight. The cells were harvested, washed twice, and used to inoculate into 3 ml of Burkholder high or low phosphate medium to an A600 of 0.05. Incubation was carried out at 30 °C for 16 h with shaking. The cells were harvested, washed with 0.05 m acetate buffer (pH 4.0), and resuspended in 1 ml of 0.05 m acetate buffer. Acid phosphatase activity was assayed according to the method described previously (16, 26).

Preparation of Cell Extracts—Cell cultures were first grown to saturation in synthetic medium lacking uracil at 30 °C. The cells were harvested by centrifugation and resuspended with sterile water, washed twice, and then incubated into the Burkholder low phosphate medium at an A600 of 0.05. These cultures were grown at 30 °C with shaking for 8 to 16 h to a final A600 of 0.5–1.5 before analysis. 50 ml of these cultures were washed with 10 ml of sterile water. All subsequent steps were carried out at 4 °C. Cells were resuspended in 450 ml of HSB buffer containing 1.0 mM PMSF, POS-ROHS (pH 5.5), 400 μm NaN, 10% glycerol, 1 mM EDTA, 0.5% Nonidet P-40, 2 mM dithiothreitol, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 4 μg/ml antipain, 10 μm NaN, 80 μm β-glycerol phosphate), and 0.8 mg of acid-washed glass beads (425–600 μm, Sigma) were added. Cells were lysed on a Vortex (Vortex-Genie II) for 30 s at the maximum setting, followed by cooling in an ice bath for 30 s. This procedure was repeated 10 times. Lysates were clarified by centrifugation at 4 °C for 15 min at 14,000 rpm twice.

Expression in Escherichia coli and Purification of GST-PHO2 Fusion Protein and PHO4 Protein—Purification of recombinant GST-PHO2 protein and its mutants was performed essentially as described (28). BL21 (DE3)-pLYsE E. coli cells (29) harboring the appropriate expression plasmids (grown in 50 ml of LB containing ampicillin (50 μg/ml) overnight at 37 °C. This culture was diluted into 500 ml of LB containing ampicillin (50 μg/ml) and grown at 30 °C until the A600 was approximately 0.5. Isopropyl-β-D-thiogalactopyranoside (Roche Molecular Biochemicals) was added to a final concentration of 1 mM, and the incubation was continued for 3 h at 30 °C. The following procedures were performed at 4 °C with ice-cold buffers. After the cells were centrifuged, the cell pellet was washed with 20 ml of phosphate-buffered saline (PBS) (150 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4, 10% glycerol (pH 7.3)). Cells were resuspended in 10 ml of PBS + 1% Triton X-100. Cell suspension was frozen in liquid nitrogen for 15 min and then frozen in –70 °C for 1 h. Frozen cells were thawed as quickly as possible at room temperature. Cell lysate was clarified by centrifugation at 14,000 rpm for 15 min 3 times. Then the cell lysate was loaded on a 1-ml glutathione-Sepharose 4B (Amersham Pharmacia Biotech) column equilibrated in PBS + 1% Triton X-100. After loading, the column was washed with 2 × 5 ml of PBS + 1% Triton X-100, then with 3 × 5 ml of PBS. The bound fusion protein was eluted with 5 ml of elution buffer (10 mM glutathione in 50 mM Tris-HCl (pH 8.0), 0.5-ml fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis.

Bl21 (DE3)-pLYsE cells harboring the PHO4 expression vector pBL-PHO2 were grown in 4 ml of LB containing ampicillin (50 μg/ml) overnight at 30 °C. The culture was diluted into 400 ml of LB containing ampicillin (50 μg/ml) and grown to A600 of 0.5 at 30 °C, then induced for 3 h at 42 °C. The following procedures were performed as described (11). Site-directed Mutagenesis of PHO4—For kinase reaction, 1 μl of YPH499 cell extract was added with 19 μl of kinase reaction mixture (20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 4 μg GST-PHO2 wild type or Pro-231 to Ser mutant), 50 μM ATP, 10 μM of (γ-32P)-ATP (Amersham Pharmacia Biotech). The reaction mixtures were incubated at 30 °C for 20 min. The reaction was stopped by the addition of 20 μl 2 × SDS sample buffer (30) and heating to 95 °C for 5 min. Denatured samples were electrophoresed on SDS, 10% polyacrylamide gel, vacuum dried, and autoradiographed.

β-Galactosidase Filter Assays—The yeast two-hybrid system was detailed in Matchmaker two-hybrid system protocol (CLONTECH (PT1265-1)). pGB9-TDC28 and pGAD424-PHO2 as well as combinations of vectors and the two-hybrid constructions were cotransformed into SFY526. Transformants were grown on synthetic medium agar plates lacking Trp and Leu at 30 °C for 2–4 days. Some clones were spotted onto nitrocellulose filters placed on synthetic medium (Trp-Leu) agar plate. The plate was then placed at 30 °C for 2 days. The filter was subsequently removed, snap-frozen on an aluminum foil case in a liquid nitrogen bath, and placed on Whatman 3MM paper saturated with Z buffer (31) containing 0.33 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The filter was incubated at 30 °C for the disappearance of blue color.

Immunoprecipitation and Protein Kinase Assay—Yeast cell extracts (in any given experiment all samples were normalized to contain the same amount of total protein (32)) were immunoprecipitated with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals) (2 μg in each reaction) for 1 h on ice. After centrifugation at 12,000 rpm for 2 min, the supernatant was added to 30 μl of slurry of protein

1 The abbreviations used are: Ycp, yeast centromeric plasmid; kb, kilobase pair; HA, hemagglutinin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; BD, binding domain; AD, activation domain.
A-agarase (Roche Molecular Biochemicals) equilibrated in HSB buffer followed by a 2-h rotation at 4 °C. Immunoprecipitates were washed with HSB buffer three times. Histone H1 kinase assays were performed as described previously (33). For phosphorylation of GST-PHO2, immunoprecipitates were washed twice with kinase assay buffer (20 mM Tris-HCl [pH 7.5], 10 mM MgCl2) and resuspended in 20 μl of kinase assay mixture (20 mM Tris-HCl [pH 7.5], 10 mM MgCl2, 4 μg of GST-PHO2, 50 μM ATP, 10 μCi of [γ-32P]ATP (Amersham Pharmacia Bio- tech)). These kinase reaction mixtures were incubated at 30 °C for 20 min. Reactions were stopped by addition of 20 μl of 2× SDS sample buffer and heating to 95 °C for 5 min. Denatured samples were electrophoresed on SDS, 10% polyacrylamide gel, vacuum-dried, and autoradiographed.

**Liquid Culture β-Galactosidase Assay**—Cell cultures were first grown to saturation in selective synthetic medium at 30 °C. The cells were harvested and washed twice with sterilized water and then incubated into 3 ml of Burkholder low (or high) phosphate medium at an A600 of 0.2. Cultures were grown at 30 °C with shaking to a final A600 of 1.5. The cells were harvested and washed twice with Z buffer. Cells were resuspended in 0.6 ml of Z buffer. 100 μl of cell suspension was removed to a fresh microcentrifuge tube. Assays for β-galactosidase activity were then performed as described in the protocol described in the MATCHMAKER two-hybrid system (PT1265-1) (CLONTECH).

**BiACore® Analysis**—Purified PHO4 protein (30 nM in 10 mM sodium acetate, pH 4.5) was injected at 5 μl/min on N-hydroxysuccinimide/N-ethyl-N’-(3-diethylaminopropyl)carbodi-imide-activated CM chips. Unreacted groups on the chip were then inactivated by 1 mM ethanol-amino-HCl, pH 8.5. A surface density of 2900 resonance units was generated in the MATCHMAKER two-hybrid system (PT1265-1) (CLONTECH).

**RESULTS**

**Mutations in the Consensus Sequence (SPIK) Recognized by cdc2/CDC28-type Kinase in PHO2 Led to Complete Loss of Its Activity**—A survey of potential recognition sites in PHO2 protein for cdc2/CDC28-type kinase or cyclic AMP-dependent protein kinase (protein kinase A) revealed a SPIK (230–233) site approximately 90-kDa protein was observed to be phosphorylated, the phosphorylation of Lys-233 to Ala showed no effect on PHO2 function to activate PHO5 expression. Intriguingly, the alteration of Lys-233 to Ala showed no effect on PHO2 function to activate PHO5 expression.

**In Vitro Phosphorylation of GST-PHO2 Fusion Protein by YPH499 Cell Extract**—To ascertain whether Ser-230 can be phosphorylated, the E. coli-expressed GST-PHO2 and GST-PHO2 (Pro-231 → Ser) were subjected to in vitro phospho-labeling experiments. The labeled proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. When the cell extract from a wild type YPH499 strain grown under low phosphate conditions was used as the substrate, an approximate 90-kDa protein was observed to be phosphorylated (Fig. 2B, lane 2), but no phosphorylated band was found at the corresponding position when GST-PHO2 (Pro-231 → Ser) was used as the substrate under identical labeling conditions (Fig. 2B, lane 2) nor when fusion protein was absent (Fig. 2B, lane 1). These results indicated that the PHO2 portion (wild type but not PHO2 (Pro-231 → Ser)) of the fusion protein is the target of the protein kinase in cell extract from YPH499 strain grown under low phosphate conditions, and the Pro-231 to Ser mutation diminished or prevented the phosphorylation of PHO2 protein. Furthermore, we also found that GST-PHO2 could be phosphorylated by cell extract from YPH499 cells.
Effects of PHO2 and PHO2 mutants on the repressive acid phosphatase of \textit{S. cerevisiae}

The wild type PHO2 gene and the PHO2 genes mutated in the potential phosphorylation sites carried by pRS415 (YCp) were introduced into the PHO2-disrupted host, YJ1 (YPH499 [pho2::URA3]). Acid phosphatase activities of the transformants were measured as described under “Experimental Procedures.” Values are the means for triplicate determinations. wt, wild type.

| Strains | Gene in YCp Vector | Acid phosphatase activity |
|---------|--------------------|--------------------------|
| YPH499(pho2::URA3) | - | 7.8 ± 0.6 |
| YPH499(pho2::URA3) PHO2(wt) | - | 227.5 ± 16.4 |
| YPH499(pho2::URA3) PHO2(S111A) | - | 224.3 ± 12.7 |
| YPH499(pho2::URA3) PHO2(S230A) | - | 9.0 ± 2.9 |
| YPH499(pho2::URA3) PHO2(P231S) | - | 11.9 ± 2.5 |
| YPH499(pho2::URA3) PHO2(K233A) | - | 270.5 ± 27.8 |
| YPH499(pho2::URA3) PHO2(S230D) | - | 255.2 ± 34.4 |

grown under high phosphate conditions (data not shown). These data allowed us to suggest that PHO2 protein can be phosphorylated in \textit{vitro} by a protein kinase(s) from YPH499 cells, and the Ser-230 may be the major phosphorylation site.

**Protein Kinase CDC28 Can Interact with PHO2 in the Two-hybrid System**—Because the potential phosphorylation site of PHO2 resembles the consensus motif of sites phosphorylated by cd2/cDC28-type protein kinase, yeast CDC28 protein was fused to the GAL4 binding domain (BD) (pGBT9-CDC28) and tested directly for interaction with GAL4 activation domain (AD)-PHO2 (expressed by pGAD424-PHO2) in the two-hybrid assay. Interaction of the two proteins was determined for their ability to activate transcription of a GAL1::\textit{lacZ} reporter gene in SFY526 by \beta-galactosidase filter assays (Fig. 3). Clones containing pGBT9-CDC28 and pGAD424 or pGBT9 and pGAD424-PHO2 were white, whereas clones containing pGBT9-CDC28 and pGAD424-PHO2 displayed a distinct blue color (Fig. 3). These results showed that CDC28 protein and PHO2 protein can interact with each other in the two-hybrid system.

**Immunoprecipitate of CDC28HA from Low Phosphate Cell Extract Can Phosphorylate GST-PHO2**—The interaction between CDC28 and PHO2 in the two-hybrid system suggested that CDC28 may be the protein kinase of the transcriptional factor PHO2. We fused an HA tag to the C terminus of CDC28 by polymerase chain reaction for the facility of immunoprecipitation. To ascertain whether CDC28 can phosphorylate PHO2 in \textit{vitro}, we used anti-HA to immunoprecipitate CDC28 from the strain YPH499 bearing CDC28HA on a multicopy expression plasmid (pVTU102-CDC28HA) grown under low phosphate conditions. The immunoprecipitated CDC28HA could phosphorylate GST-PHO2 (Fig. 4A, lane 1), whereas the immunoprecipitate from control strain YPH499 containing vector pVTU102 had very low kinase activity (Fig. 4A, lane 2). The immunoprecipitate derived from cell extract containing CDC28HA phosphorylated histone H1 (Fig. 4B, lane 1), whereas the control had only background kinase activity (Fig. 4B, lane 2). These results showed that PHO2 could be phosphorylated in \textit{vitro} by CDC28 kinase complex from YPH499 (pVTU102-CDC28HA) grown under low phosphate conditions.

**Phosphate Switch Regulates the Transcriptional Activation Activity of PHO2 and Mutations of the (SPIK) Site Affect the Transcriptional Activation Activity of PHO2 and the Interaction between PHO2 and PHO4—**PHO2 was fused to the DNA BD of yeast transcriptional factor GAL4. The plasmid expressing GAL4 BD-PHO2 chimera, pGBT9-PHO2, was transformed into a yeast strain, SFY526, containing an integrated \textit{GAL1::lacZ} reporter gene. By measuring the expression of the reporter gene (\textit{lacZ}) under high and low phosphate conditions (Table II), it was found that the chimera alone could activate the expression of \textit{lacZ}, and the \beta-galactosidase activity was regulated by the phosphate switch. The activity under low phosphate condition was 8-fold more than that under high phosphate conditions. In contrast, when pVA3 (containing the fusion of murine p53 and GAL4 DNA binding domain) and pTD1 (containing a fusion of SV40 large T-antigen and GAL4 activation domain) were cotransformed into SFY526, the expression of \textit{lacZ} was also activated, but the \beta-galactosidase activity was not affected by the phosphate switch. These results demonstrated that the transcriptional activation activity of PHO2 was regulated by the phosphate switch, which was repressed under high phosphate condition and was derepressed under low phosphate conditions.

The SPIK (230–233) site near PHO80 homologous region is a potential phosphorylation site of PHO2, and the phosphorylation of Ser-230 is necessary to activate PHO5 transcription. The mutation of Ser-230 to Ala resulted in the loss of ability to be phosphorylated, so GAL4BD-PHO2 (Ser-230 → Ala) chimera could not activate the expression of \textit{lacZ} irrespective of phosphate concentration (Table II). The substitution of Asp for Ser-230, which mimics the phosphorylation state by providing phosphate switch, was not affected by the phosphate switch. These results showed that the

### Table I

| Strains | Gene in YCp Vector | Acid phosphatase activity |
|---------|--------------------|--------------------------|
| YPH499(pho2::URA3) | - | 7.8 ± 0.6 |
| YPH499(pho2::URA3) PHO2(wt) | - | 227.5 ± 16.4 |
| YPH499(pho2::URA3) PHO2(S111A) | - | 224.3 ± 12.7 |
| YPH499(pho2::URA3) PHO2(S230A) | - | 9.0 ± 2.9 |
| YPH499(pho2::URA3) PHO2(P231S) | - | 11.9 ± 2.5 |
| YPH499(pho2::URA3) PHO2(K233A) | - | 270.5 ± 27.8 |
| YPH499(pho2::URA3) PHO2(S230D) | - | 255.2 ± 34.4 |

Fig. 2. **In vitro phosphorylation of GST-PHO2 fusion proteins by YPH499 cell extract.** A, purified GST-PHO2 (lane 2) and the mutant GST-PHO2 (Pro-231 to Ser) (lane 3) were analyzed by 10% SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. B, the wild type PHO2 (lane 2) and the mutant PHO2 (Pro-231 to Ser) (lane 3) fused with GST were subjected to \textit{in vitro} phospho-labeling experiments using cell extract of YPH499 grown under low phosphate conditions of enzyme as described under “Experimental Procedures.” Fusion protein was absent in lane 1. Phosphorylation proteins were visualized by 10% SDS-polyacrylamide gel electrophoresis and autoradiography.

Fig. 3. **Interaction between CDC28 and PHO2 in yeast two-hybrid system.** pGBT9 contains a GAL4 DNA binding domain. pGAD424 contains a GAL4 activation domain. pGBT9-CDC28 contains the fusion of CDC28 and GAL4 DNA binding domain. pGAD424-PHO2 expresses GAL4AD-PHO2 chimera. pVA3, murine p53 in pGBT9. pTD1 contains the fusion of SV40 large T-antigen and GAL4 activation domain. The cotransformant of pTD1 and pVA3 was a positive control. \beta-Galactosidase activity of SFY526 transformants was assayed as described under “Experimental Procedures.” Each was repeated twice as shown.
Phosphorylation of PHO2

When pGBT9-PHO2 alone or plasmid 1 and plasmid 2 as shown in the table. The level of β-galactosidase activity derived from a GAL1-lacZ reporter was determined as described under “Experimental Procedures.” Values are the means for triplicate determinations. pGBT9-PHO2 and pGBT9-PHO2 (S230A) contain the fusions of PHO4 or PHO2 (S230A) mutant and GAL4 DNA-binding domain. pGAD424-PHO4 contains the fusion of PHO2 and GAL4 activation domain. pGBT9-PHO4 and pGAD424-PHO4 contain the fusions of PHO4 and GAL4 DNA-binding domain or activation domain. pVA3, murine p53 in pGBT9. pTD1 contains the fusion of SV40 large T-antigen and GAL4 activation domain.

**TABLE II**

Analysis of transcriptional activation activity of yeast PHO2 protein

| Plasmid 1 | Plasmid 2 | β-Galactosidase activity |
|-----------|-----------|--------------------------|
| pGAD424   | pVA3      | High P1 | pPD1 | Low P1 |
| pGBT9-PHO2| pGAD424-PHO4| 15.9 ± 1.7 | 38.9 ± 9.0 |
| pGBT9-PHO2(S230A)| pGAD424| 43.9 ± 3.3 | 219.0 ± 7.7 |
| pGBT9-PHO4| pGAD424-PHO4| 89.4 ± 9.3 | 306.4 ± 18.0 |
| pGBT9-PHO2(S230A)| pGAD424-PHO4| 0 | 0 |

FIG. 4. CDC28 phosphorylates PHO2 in vitro. A, phosphorylation of GST-PHO2 assay. The phosphorylation of GST-PHO2 was assayed by immunoprecipitates from the strain YPH499, bearing CDC28HA on a multicopy expression plasmid (pVTU102-CDC28HA) (lane 1), and a control strain YPH499 containing the empty plasmid (pVTU102) (lane 2). B, histone H1 kinase assay. The immunoprecipitates from the strain YPH499 containing CDC28HA (lane 1) and the control strain (lane 2) was used in histone H1 kinase assay.

phosphorylation of PHO2 Ser-230 is necessary for the activation activity of PHO2. The phosphorylation site-containing negative charge may resemble an acidic activation domain. However, the activity of GAL4BD-PHO2 (S230D) chimera was also regulated by the phosphate switch. The activity under low phosphate condition was 5-fold more than that under high phosphate condition.

The expression of lacZ could also be activated by GAL4BD-PHO2 chimera in another strain, SFY526 (pho4::HIS3). The activity under low phosphate conditions was 3-fold more than that under high phosphate condition (Table III), although it was less than that in SFY526. When pGBT9-PHO2 was co-transformed with pRS4 (YCp containing a PHO4 gene), it had no significant effect on the β-galactosidase activity under high phosphate condition, but the activity under low phosphate conditions was 1-fold more than that using the GAL4BD-PHO2 chimera alone (Table III). The results demonstrated that these two proteins may interact with each other. When pGBT9-PHO4 (for expression of GAL4BD-PHO4 chimera) was cotransformed into SFY526 (pho4::HIS3) with pGAD424-PHO2 (for expression of GAL4 AD-PHO2 chimera), the β-galactosidase activity was 40% more than that using pGBT9-PHO4 and pGAD424. The result also indicated that PHO4 and PHO2 may interact in vivo.

Another result showed that the phosphorylation of PHO2 is necessary for the interaction between PHO2 and PHO4. When GAL4BD-PHO2 (S230A) was coexpressed with GAL4AD-PHO2, the phosphorylation of PHO2 Ser-230 is necessary for the interaction between PHO2 and PHO4. When GST-PHO2 (S230D) was treated with CDC28 protein, which mimics the phosphorylation of Ser-230, may help the interaction between PHO2 and PHO4, which may play a key role in maintaining the transcriptional activity of PHO2.

**TABLE III**

Two hybrid assay of PHO2 and PHO4 protein

| Plasmid 1 | Plasmid 2 | β-Galactosidase activity |
|-----------|-----------|--------------------------|
| pGAD424   | pVA3      | High P1 | pPD1 | Low P1 |
| pGBT9-PHO2| pGAD424-PHO4| 11.0 ± 0.2 | 38.9 ± 9.0 |
| pGBT9-PHO2(S230A)| pGAD424| 15.9 ± 1.7 | 79.9 ± 14.0 |
| pGBT9-PHO4| pGAD424-PHO4| 43.9 ± 3.3 | 219.0 ± 7.7 |
| pGBT9-PHO2(S230A)| pGAD424-PHO4| 89.4 ± 9.3 | 306.4 ± 18.0 |
| pGBT9-PHO2(S230A)| pGAD424-PHO4| 0 | 0 |

BIAcore® Analysis of the Interaction between PHO2 and PHO4 in Vitro—The data above showed that the phosphorylation of PHO2 Ser-230 is necessary for the interaction between PHO2 and PHO4. To investigate whether the phosphorylation of Ser-230 is sufficient to mediate interaction between PHO2 and PHO4, we used purified GST-PHO2, GST-PHO2SA (Ser230 → Ala), GST-PHO2SD (Ser230 → Asp), and PHO4 for BIAcore® analysis.

PHO4 was covalently linked to a sensor chip (CM5). GST-PHO2, GST-PHO2SA, and GST-PHO2SD, at the same concentration of 5 μM, were injected over the surface. As shown by the sensorgrams obtained (Fig. 5), a strong signal was detected when GST-PHO2SD was injected (Fig. 5B), whereas signals were very weak when GST-PHO2 (Fig. 5A) or GST-PHO2SA (Fig. 5C) was used. The sensorgram of GST-PHO2SD showed a very typical interaction. The effect of impurities and the fused GST was eliminated for the results of GST-PHO2 and GST-PHO2SA. The results indicated the negative charge of PHO2 (Ser-230 → Asp), which mimics the phosphorylation of Ser-230, may help the interaction between PHO4 and GST-PHO2SD.

**DISCUSSION**

The functions of many transcriptional factors are regulated by phosphorylation and dephosphorylation, which may act in several aspects. First, phosphorylation can change the cellular location of some transcriptional factors. An S. cerevisiae transcriptional factor, SWI5, which is localized in the cytoplasm at the G2 phase of cell cycle due to its phosphorylation by the cyclin-CDC28 protein kinase, enters the nucleus at Start in the G1 phase and activates HO gene expression (35). Similarly, PHO4 is localized in the cytoplasm under high phosphate conditions (38) due to hyperphosphorylation by the PHO80-PHO85 cyclin-CDK (CDK, cyclin-dependent kinase) (11). Second, some transcriptional activation factors, such as GAL4 and GCN4 (37, 38), which have acidic transcriptional activation domains, may have a higher transcriptional activation activity upon point mutations to increase negative charges. It is conjectured that phosphorylation and dephosphorylation may regulate the transcriptional activation activity by the increase or decrease of negative charges in response to the change of the circumstance.
Phosphorylation of PHO2

Third, phosphorylation may affect the interaction between transcriptional factors. Here we found that phosphorylation of PHO2 can affect the transcriptional activation activity. Only when PHO2 is phosphorylated, the secreted acid phosphatase, PHO5, can be expressed under low phosphate conditions. Further investigations indicated that the phosphorylation of PHO2 not only improve the transcriptional activation activity but also facilitates the interaction between PHO2 and PHO4. These results suggested the pleiotropy of PHO2 phosphorylation. How the critical phosphorylation contributes to transcriptional activation activity and interaction is unclear. Because the previous deletion analysis showed that the possible PHO4 binding domain of PHO2 does not include the SPIK site (13, 17) and our results indicated that the negative charge of PHO2 (Ser230 → Asp) is sufficient to help the interaction between PHO2 and PHO4 in vitro, it suggests that the phosphorylation of the Ser-230 residue in PHO2 may alter the conformation of PHO2, making the PHO4 binding domain of PHO2 suitable for interaction with PHO4 but not participating in the interaction directly. The phosphorylation may also increase the ability of PHO2 to activate transcription by altering the three-dimensional conformation of PHO2. Furthermore, the phosphate group may be directly involved in transcriptional activation by increasing the negative charges of activation domain of PHO2.

PHO2 has a potential phosphorylation site, SPIK (230–233), that resembles the consensus sequence of site recognized and phosphorylated by cdc2/CDC28-type kinase (20–23). We found that GST-PHO2 fusion protein expressed in E. coli could be phosphorylated by yeast cell extract from YPH499 strain grown under low phosphate conditions. Further investigation showed that CDC28HA immunoprecipitate derived from yeast grown under low phosphate conditions could phosphorylate GST-PHO2 in vitro. These results indicate that CDC28 may be the kinase of PHO2. CDC28 is an essential regulator of cell cycle progression, whereas PHO2 is required for several metabolism processes. Another yeast cyclin-dependent kinase, PHO85, has provided a link between cell cycle regulation and phosphate metabolism (11, 39, 40). By association with different cyclin subunits, PHO85 may be directed to distinct biological functions. If CDC28 could be proved to be the physiological kinase of PHO2, another link between cell cycle progression and metabolism would be found. Perhaps a new cyclin would be found to help CDC28 participate in metabolism. In addition to PHO85, CDC28 might also serve to coordinate nutritional state and cell cycle progression.

Homeo box-encoding genes appear to be associated with developmental control or cell type regulation. Previous investigations carried out by Gilliquet and Berben (41) reveals the involvement of PHO2 that contains a homeo domain in the life cycle. Homozygous PHO2 null diploids show a deficiency to progress through meiosis, which can be corrected when the cells are transformed with PHO2-bearing plasmid. Many nuclear factors involved in the cell cycle have been observed to be phosphorylated and regulated by cdc2/CDC28-type protein kinase, which is activated only at specific cell cycle stages (42). Our present findings show that PHO2 may be phosphorylated by CDC28. These evidences also suggest that there is some association between metabolism and cell cycle machinery.

FIG. 5. Sensorgrams of GST-PHO2SD protein binding to immobilized PHO4 protein in BLAcore® experiments. PHO4 protein was covalently linked to a sensor chip (CM5). 5 μM concentrations of GST-PHO2 (A), GST-PHO2SD (Ser-230 → Asp) (B), and GST-PHO2SA (Ser-230 → Ala) (C) were injected over the surface of the sensor chip (see “Experimental Procedures” for details). RU, resonance units.

REFERENCES

1. Yoshida, K., Ogawa, N., and Oshima, Y. (1989) Mol. Gen. Genet. 217, 40–46
2. Arndt, K. T., Styles, C., and Fink, G. R. (1987) Science 237, 874–880
3. Sengstag, C., and Hinnen, A. (1987) Nucleic Acids Res. 15, 233–246
4. Vogel, K., Horz, W., and Hinnen, A. (1989) Mol. Cell. Biol. 9, 2050–2057
5. Wu, J. S., Chen, J. L., and Ao, S. Z. (1996) Acta Biochim. Biophys. Sin. 28, 507–515
6. Sengstag, C., and Hinnen, A. (1988) Gene 67, 223–228
7. Braus, G., Mosch, H. U., Vogel, K., Hinnen, A., and Hutter, R. (1989) EMBO J. 8, 939–945
8. Brazas, R. M., and Stillman, D. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11237–11241
9. Daum-Fournier, B., and Fink, G. R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6746–6750
10. Oshima, Y. (1991) The Molecular Biology of Yeast Saccharomyces: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W., and Broach, J. R., eds) pp. 159–180, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
11. Kaufman, A., Herskowitz, I., Tjian, R., and O’Shea, E. K. (1994) Science 263, 1153–1156
12. Schneider, K. R., Smith R. L., and O’Shea, E. K. (1994) Science 266, 122–126
13. Yang, J., Wu, J. S., and Ao, S. Z. (1995) Acta Biochim. Biophys. Sin. 27, 165–172
14. Burgin, T. R. (1986) Cell 52, 339–340
15. Bergen, B., Legrain, M., and Hilger, F. (1988) Gene 66, 307–312
16. Hurst, K., Fisher, F., McAndrew, P. C., and Cogdill, G. R. (1994) EMBO J. 13, 5410–5420
17. Shao, D., Creasy, C. L., and Bergan, L. W. (1996) Mol. Gen. Genet. 251, 358–364
18. Magbanua, J. D., Ogawa, N., Harashima, S., and Oshima, Y. (1997) J. Biochem. (Tokyo) 121, 1182–1189
19. Matsumoto, K., Uno, I., and Ishikawa, T. (1984) Genetics 108, 53–56
20. Hill, C. S., Packman, L. C., and Thomas, J. O. (1990) EMBO J. 9, 805–813
21. Ward, G. E., and Kirschner, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 507–515
22. Moreno, S., and Nurse, P. (1990) Cell 51, 549–551
23. Pearson, R. B., and Kemp, B. E. (1991) Methods Enzymol. 200, 62–81
24. Gietz, R. D., and Schiestl, R. H. (1995) Mol. Cell. Biol. 5, 255–269
25. Burkholder, P. R. (1943) Am. J. Bot. 29, 459–464
26. Toh-e, A., Ueda, Y., Kakimoto, S., and Oshima, Y. (1973) J. Bacteriol. 113, 727–738
27. Verne, T., Dignard, D., and Thomas, D. Y. (1987) EMBO J. 6, 325–332
28. Becker, D., Breit, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, D. B., and Jahson, K. S. (1986) Proc. Natl. Acad. Sci. U. S. A. 89, 6139–6143
29. Studier, F. W. (1991) J. Mol. Biol. 219, 37–44
30. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, D. B., and Jahson, K. S. (1986) Proc. Natl. Acad. Sci. U. S. A. 89, 6139–6143
31978

Phosphorylation of PHO2

J. A., and Struhl, K. (eds) (1987) Current Protocols in Molecular Biology, Wiley Interscience, New York
31. Harshman, K. D., Moyer-Roeley, W., and Parker, C. S. (1988) Cell 53, 321–330
32. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
33. Jeoung, D. I., Oehlen, L. J., and Cross, F. R. (1998) Mol. Cell. Biol. 18, 433–441
34. Zetter, O., Ragnarsson, U., and Engstrom, L. (1990) in Peptides and Protein Phosphorylation (Kemp, B. E., ed) pp. 171–187, CRC Press, Inc., Boca Raton, FL
35. Feaver, W. J., Henry, N. L., Bushnell, D. A., Sayre, M. H., Brickner, J. H., Gileadi, C., and Kornberg, R. D. (1994) J. Biol. Chem. 269, 27549–27553
36. O’Neill, E. M., Kaffman, A., Jelly, E. R., and O’Shea, E. K. (1996) Science 271, 209–212
37. Ma, J., and Ptashne, M. (1987) Cell 48, 847–853
38. Hope, I. A., and Struhl, K. (1986) Cell 46, 885–894
39. Espinoza, F. H., Ogas, J., Herskowitz, I., and Morgan, D. O. (1994) Science 266, 1388–1391
40. Meadlay, V., Moore, L., Ogas, J., Tyers, M., and Andrews, B. (1994) Science 266, 1392–1395
41. Gilliquet, V., and Berben, G. (1993) FEMS Microbiol. Lett. 108, 333–340
42. Forsburg, S. L., and Nurse, P. (1991) Annu. Rev. Cell Biol. 7, 227–256