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The Transcriptional Activators of the *PHO* Regulon, Pho4p and Pho2p, Interact Directly with Each Other and with Components of the Basal Transcription Machinery in *Saccharomyces cerevisiae*[1]

Jose Paolo V. Magbanua, Nobuo Ogawa, Satoshi Harashima, and Yasuji Oshima[2]

Department of Biotechnology, Faculty of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565

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The transcriptional regulators Pho4p and Pho2p are involved in transcription of several genes in the *PHO* regulon of *Saccharomyces cerevisiae*. Genetic evidence with temperature-sensitive *pho4* and *pho2* mutants suggested that Pho4p and Pho2p interact with each other. Immunoprecipitation experiments showed that Pho4p and Pho2p form a complex on a 36-bp sequence bearing an upstream activation site (UAS) and protein binding assays indicated that these proteins interact directly. DNA-binding experiments with crude extracts prepared from yeast strains expressing *T7-PHO4*, encoding Pho4p tagged with the T7 epitope, indicated that Pho2p interacts with T7-Pho4p and enhances the binding affinity of T7-Pho4p to the UAS. Protein binding experiments also showed that both Pho4p and Pho2p could bind with the general transcription factors, TBP, TFIIB, and TFIIEβ, suggesting that the Pho4p-Pho2p complex bound to the UAS activates transcription of the *PHO* genes by direct interaction with the general transcription factors.

Key words: *PHO* regulon, Pho2p, Pho4p, transcriptional regulator, yeast.

In the yeast *Saccharomyces cerevisiae*, transcription of *PHO5* encoding the major fraction of repressible acid phosphatase [EC 3.1.3.2], *PHO8* encoding a repressible alkaline phosphatase [EC 3.1.3.1], *PHO84* encoding a P_i-transporter, and *PHO81* encoding one of the regulatory factors in the *PHO* regulon is negatively regulated by P_i in the medium [for a review, see Oshima *et al.* (*1*)]. In low P_i medium, the positive regulatory protein Pho4p binds to its target upstream activation sites (UASs) together with another regulatory protein, Pho2p, and activates transcription of *PHO5*, *PHO81*, and *PHO84*, with the exception of *PHO8*, whose expression occurs with Pho4p alone (*2*). Under high P_i conditions, the activity of Pho4p is blocked *via* hyperphosphorylation by a complex of Pho80p (cyclin) and Pho85p (protein kinase) (*3*), as the hyperphosphorylated Pho4p cannot enter the nucleus (*4*). In the low-P_i medium, Pho81p inhibits the function of the Pho80p-Pho85p complex (*5*) under the influence of P_i concentration in the medium, possibly through the function of two putative membrane proteins, Pho86p and Pho88p, associated with the Pho84p P_i-transporter (*6*).

It was predicted based on the nucleotide sequence of *PHO4* that Pho4p has a basic helix-loop-helix structure at its C-terminal region as the DNA binding domain and an acidic sequence at its N-terminal region functions as the transcriptional activation domain (*7*). We found two types of 9-bp UAS motifs, 5′-GCACGTGGG-3′ and 5′-GCACGTTTT-3′, for binding Pho4p in the promoters of various *PHO* structural genes (*8*). The other regulatory protein, Pho2p, is a homeodomain protein (*9*) required for proper regulation of *PHO5*, *PHO84*, and *PHO81*, but is dispensable for *PHO8* regulation. It also regulates basal expression of *HIS4* (*10*), *ADE1*, *ADE2*, *ADE5,7*, and *ADE8* together with Bas1p (*11*) and modulates amino acid synthesis in response to amino acid and phosphate starvation (*12*). It also binds cooperatively with Swi5p to activate *HO* expression (*13*).

Based on the results of two-hybrid assay, Hirst *et al.* (*14*), however, argued that DNA binding by Pho4p is dependent on the phosphate-sensitive interaction with Pho2p. It was also suggested that interaction of Pho2p with Pho4p increases the accessibility of the activation domain of Pho4p (*15*). Recently, DNase I footprinting and gel-retardation assays indicated that Pho4p and Pho2p form a ternary complex at the *PHO5* promoter (*16*). It is not clear, however, how Pho2p influences Pho4p and what are the consequences of ternary complex formation.

It has been proposed that eukaryotic transcriptional regulators bound to the promoter region of a gene induce the assembly of a pre-initiation complex containing RNA polymerase II and several transcription factors at a site near the transcription start site (*17*). These regulators were suggested to initiate transcription through specific interactions with basal transcription factors and TBP-

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[2] To whom correspondence should be addressed at the present address: Department of Biotechnology, Faculty of Engineering, Kansai University, 3-3-35 Yamatecho, Suita, Osaka 564. Tel: +81-6-368-0815, Fax: +81-6-388-8609, e-mail: oshima@ipcku.kansai-u.ac.jp

Abbreviations: aa, amino acid(s); Amp^R, ampicillin-resistant phenotype; GST, glutathione *S*-transferase; His, histidine; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria-Bertani (broth); OD_{600} and OD_{660}, optical density at 600 nm and 660 nm, respectively; ORF, open reading frame; PMSF, phenylmethylsulfonyl fluoride; TBP, TATA binding protein; *ts* (superscript), temperature-sensitive; UAS, upstream activation site.

associated factors (*18*). Various transcriptional regulators have been shown to bind directly with TBP and/or TFIIB (*19, 20*).

Here we show that direct protein-protein contact occurs between Pho2p and Pho4p and that this interaction results in increased binding affinity of Pho4p to the UAS. Protein binding assays indicated that both Pho4p and Pho2p could bind with the basal transcription factors TBP, TFIIB, and TFIIEβ. This suggests that the *PHO* gene transcription is initiated by direct contacts between the Pho4p-Pho2p complex and the basal transcription factors.

MATERIALS AND METHODS

Organisms and Plasmids—The *S. cerevisiae* strains used are listed in Table I. *Escherichia coli* strains TG1 and DH5α (*23*) were used as hosts for DNA manipulation and BL21(DE3) (*24*) along with DH5α were used as hosts for protein production.

Plasmid pYS104 marked with *LEU2* and used to express a *T7-PHO4* fusion gene in yeast was constructed as follows: Plasmid pACT7-PHO4 (*25*) was digested with *Nde*I, filled-in using the Klenow fragment, and digested with *Mlu*I. The resultant 0.6-kb *Nde*I-*Mlu*I fragment was used to replace a similar 0.6-kb *Bam*HI (filled in)-*Mlu*I region of the L-3 fragment (*7*) bearing the *PHO4* ORF ligated in YEp131. The resultant plasmid, pYS104, produces Pho4p in which an 11-aa T7 epitope, MASMTGGQQMG, is inserted between a 6-aa sequence, MGRPGS, and RDP at the N-terminal of Pho4p. Another *T7-PHO4* plasmid, pYS143, bearing the same *T7-PHO4* fusion gene but marked with *URA3* was constructed by inserting a 2.0-kb *Hin*dIII-*Sal*I *T7-PHO4* fragment from pYS104 into the *Hin*dIII-*Sal*I gap of YEp241 (*5*). The *T7-PHO4* fusion gene in these two plasmids is transcribed under the control of the native *PHO4* promoter.

To construct plasmid pGST-PHO4, the L-3 fragment of *PHO4* (*7*) was double-digested with *Bam*HI and *Hin*cII, and the 1.2-kb *Bam*HI-*Hin*cII fragment obtained was inserted into the *Bam*HI-*Sma*I site of plasmid pGEX-2T (Pharmacia). The resultant plasmid, pGST-PHO4, produces Pho4p with a 3-aa deletion at the N-terminal and connected with a 240-aa peptide of glutathione *S*-transferase (GST) *via* GSG residues.

TABLE I. **The *Saccharomyces cerevisiae* strains used.**

Strain	Genotype	Source
NBW7	*MAT*a *pho3-1 ade2 leu2-3,112 his3-532 ura3-1,2 can1*	(*7*)
NBD4-4A[a]	*MAT*a *pho3-1 Δpho4::HIS3 ade2 leu2-3,112 his3-532 trp1-289 ura3-1,2 can1*	Our stock
NBD24-1A[b]	*MAT*a *pho3-1 Δpho4::HIS3 Δpho2::HIS3 ade2 leu2-3,112 his3-532 trp1-289 ura3-1, 2 can1*	Our stock
NBD480-6C[c]	*MAT*a *pho3-1Δpho4::HIS3 Δpho80::HIS3 ade2*	Our stock
HYP100	*MAT*a *ura3 leu2 trp1 his3 ade2 lys2*	(*21*)

[a]NBD4-4A is a *Δpho4* haploid segregant from an NBD4-1 (*7*) ×NBW8 [a haploid segregant from the diploid strain NBW78 (*22*)] cross. [b]NBD24-1A is a *Δpho2 Δpho4* haploid segregant from an NBD4-4A ×NBD2-1A cross. NBD2-1A is a *Δpho2* haploid segregant from an NBW8 ×NBD2-1 (*22*) cross. [c]NBD480-6C is a *Δpho4 Δpho80* haploid segregant from repeated crosses of NBD4-4A, NBW8, and NBD80-1 (*22*).

Plasmid pET21-X-PHO2 bearing a DNA fragment encoding Pho2p tagged with the T7 epitope at the N terminal and six histidine residues at the C-terminal (T7-Pho2p-His) was constructed as follows: A 12-bp *Bam*HI linker (Takara Shuzo) was ligated to the *Sna*I end of a 1.6-kb *Sna*I (position −11 from the ATG codon of the *PHO2* ORF)-*Bcl*I (at position +1579 in the downstream of the ORF) fragment bearing *PHO2* (*26*) and the resultant fragment was inserted into the *Bam*HI site of pET-3d (Novagen). Then, a 1.9-kb *Sph*I (0.2 kb upstream of the T7 promoter in pET-3d)-*Hin*cII (at position +1484 in the *PHO2* ORF) fragment was prepared from the resulting plasmid. A 4-bp *Hin*cII/*Xho*I connection, TCGA, from pBluescript II SK+ (Stratagene) was ligated to the *Hin*cII end of the 1.9-kb fragment and the resultant *Sph*I-*Xho*I fragment was inserted into the *Sph*I-*Xho*I gap of pET-21a (Novagen). The resultant plasmid, pET21-X-PHO2, produces a truncated Pho2p bearing aa residues from positions 1 to 495 [the wild-type *PHO2* ORF encodes a 519-aa protein (*26*)], but connected with the 11-aa T7 epitope at the N-terminal and having a 7-aa segment, RIRVQYA, between the T7-epitope and Pho2p. The C-terminal end of this truncated T7-Pho2p chimeric protein is connected with six histidine repeats through three aa residues, DLE.

Plasmid pPHO2-Myc was constructed by preparing a 2.5-kb *Hin*dIII-*Hin*cII fragment from the *PHO2* gene (*26*) and inserting it into the *Hin*dIII-*Sma*I gap of pYC209 (*27*). The *PHO2* gene in this plasmid is transcribed by the native *PHO2* promoter and produces a Pho2p-Myc protein composed of Pho2p truncated at the C-terminal from aa position 496 to the end, but connected with an 11-aa c-Myc epitope (*28*) *via* a proline residue.

To construct plasmid pGST-PHO2, a 1.5-kb *Bam*HI-*Hin*cII fragment was prepared from the 1.9-kb *Sph*I-*Xho*I fragment of pET21-X-PHO2 and inserted into the *Bam*HI-*Sma*I gap of plasmid pGEX-3X (Pharmacia). This plasmid produces a chimeric protein consisting of aa residues from positions 1 to 495 of Pho2p connected with a 240-aa region of GST at the N-terminal *via* an aa sequence IRVQYA and having an aa sequence GNSS at its C-terminal region.

To prepare yeast TATA binding protein (TBP), TFIIB, and TFIIEβ, connected with the T7 epitope, yeast *SPT15* gene encoding TBP (*29*) was amplified by PCR using genomic DNA of *S. cerevisiae*, prepared from strain HYP100 (Table I), as the template, and oliTBPf (5′-CTCGGATCCCCATGGCCGATGAGGAACGTTT-3′) and oliTBPr (5′-CTCGGATCCTCACATTTTTCTAAATTCAC-3′) as the primers. The amplified fragment was digested with *Bam*HI and a 0.7-kb fraction of the *Bam*HI fragments separated by PAGE was inserted into the *Bam*HI site of pET-3c (Novagen). One of the resultant plasmids, pET-TBP, produces a chimeric protein consisting of aa residues from positions 1 to 241 of TBP (full length) connected with the 11-aa T7 epitope *via* aa residues RIP. The *SUA7* gene encoding TFIIB (*30*) was also amplified by PCR using oliTFIIBf (5′-CTCAGATCTCCATGATGACTAGGGAGAGCAT-3′) and oliTFIIBr (5′-CTCAGATCTTTATTTCTTTTCAACGCCCG-3′) as the primers. The amplified DNA was digested with *Bgl*II and a 1-kb fraction of the *Bgl*II fragments was purified as above and inserted into the *Bam*HI gap of plasmid pET-3c. This plasmid, pET-TFIIB, produces full-length TFIIB connected with the 11-aa T7 epitope *via* aa residues RIS. Similarly, the *TFA2* gene

encoding the TFIIEβ subunit of TFIIE (*31*) was amplified using oliTFIIFβf (5′-CTCGGATCCCCATGAGTAAAAA-CAGGGACCC-3′) and oliTFIIFβr (5′-CTCGGATCCTCA-TACTCTATGGGAATAAT-3′) as the primers. The amplified fragments were digested with *Bam*HI and a 1-kb fraction of the *Bam*HI fragments was inserted into the *Bam*HI site of pET-3c. This plasmid, pET-TFIIEβ, produces full-length TFIIEβ connected to the 11-aa T7 epitope *via* aa residues RIP.

Media, Genetic and Biochemical Methods—LB broth, Ura and Leu test media, and synthetic low- and high-P_i media were prepared as described previously (*26*). Routine methods were used for yeast transformation (*32*). Preparation, modification, and sequencing of DNA and PAGE were performed as described (*23*). Oligonucleotides were synthesized as described (*8*). Protein concentration was determined using a protein assay kit (Bio-Rad).

Preparation of Yeast Cell Extract—Cells to be tested were inoculated into 100 ml of synthetic high- or low-P_i medium and grown at 30°C to an OD_{660} of 1.0. The cells were harvested, washed twice with extraction buffer (*33*), and collected by centrifugation, then the pellet was suspended in 0.4 ml of extraction buffer with 1 mM PMSF, and 1 μg each of aprotinin, leupeptin, and pepstatin per ml. The suspension was transferred into a microcentrifuge tube containing an equal volume of glass beads and the cells were disrupted by vortexing four times in 1 min bursts followed by 1 min cooling on ice, then centrifuged for 20 min at 13,000 rpm at 0°C in a microcentrifuge. The supernatant was transferred into a fresh tube and the protein concentration was determined.

Co-Immunoprecipitation and DNA Binding Assays—For the co-immunoprecipitation assay, 1 pmol of 36-bp fragment bearing the site D5′A UAS of *PHO84* (*8*) was added to 25 μl of 10$\times$buffer E [1$\times$buffer E is 10 mM Tris pH 7.9, 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, and 100 μg of bovine serum albumin per ml (*34*)] with 2.5 μg of calf thymus DNA per ml, instead of chick blood DNA, and 500 μg of yeast-extract protein in 200 μl of extraction buffer (*33*). The reaction mixture was incubated on ice for 1 h, then 4 μl of anti-Pho4p antibody (*5*) or 10 μl of anti-Myc monoclonal antibody (Oncogene Science) was added to the reaction mixture. After incubation of the above reaction mixture on ice for 1 h, 50 μl of Protein A Sepharose FF (Pharmacia) in buffer E was added and incubation on ice was continued for 1 h with occasional agitation. The reaction mixture was centrifuged for several seconds at 10,000 rpm in a microcentrifuge and the supernatant was discarded. The Sepharose pellet was washed three times with 0.5 ml each of IP buffer [25 mM Tris pH 7.0, 2 mM EGTA, 150 mM NaCl, 1% Nonidet P-40 (*34*)], then 50 μl of SDS-PAGE gel-loading buffer (*23*) was added and the mixture was boiled for 5 min. SDS-PAGE was done on a 10% separation gel at 6°C to separate precipitated proteins. Proteins were electroblotted onto a polyvinylidene difluoride membrane (Millipore) and analyzed by Western blotting with various antisera as described (*23*).

The method of DNA binding was the same as that of the co-immunoprecipitation assay, except that 1 pmol of ^{32}P-labeled 36-bp fragment bearing the UASp2 site of *PHO5* was added to the reaction mixture containing yeast extract (1.5 mg of protein in 200 μl of buffer E). The mixture was allowed to stand on ice for 1 h, and 3 μl of anti-T7 monoclonal antibody (Novagen) was added. The whole was mixed, and further incubated on ice for 1 h. Then 50 μl of Protein A Sepharose FF in buffer E was added and the reaction mixture was incubated on ice for 1 h with occasional agitation. It was centrifuged for several seconds at 10,000 rpm and the supernatant was discarded. The Sepharose pellet was washed three times with 0.5 ml each of IP buffer and suspended in 100 μl of buffer F [10 mM Tris pH 7.4, 20 mM NaCl, and 0.1% SDS (*34*)], then the DNA was recovered by phenol extraction and ethanol precipitation. The recovered DNA was subjected to 10% PAGE in 1$\times$TBE (89 mM Tris-borate and 25 mM EDTA) followed by drying and autoradiography of the gel.

Preparation of Chimeric Proteins—Plasmid pET21-X-PHO2 was introduced into *E. coli* BL21(DE3) and an Amp^R transformant was isolated and inoculated into LB broth supplemented with 50 μg of ampicillin per ml. The culture was shaken at 30°C to an OD_{600} of 0.6, then IPTG was added to a final concentration of 1 mM and shaking at 30°C was continued for 1 h. The purified T7-Pho2p-His sample was prepared from 600 ml of the above culture by rapid affinity purification using the His·Bind Resin and Buffer Kit (Novagen) according to the manufacturer's instructions. The T7-Pho2p-His fraction was eluted in the 3rd and 4th 0.5-ml fractions after addition of elution buffer (Novagen). The eluent was dialyzed against 1 liter of buffer T(50) (*25*). Then, the protein concentration was determined. Preparation of purified T7-Pho4p from *E. coli* has been described previously (*25*). For preparation of GST fusion proteins of Pho2p and Pho4p, a single colony of *E. coli* DH5α Amp^R transformant harboring any one of the three plasmids, pGEX-3X, pGST-PHO2, and pGST-PHO4, was inoculated into 100 ml (or 400 ml for the pGST-PHO2 transformant) of LB supplemented with 50 μg of ampicillin per ml and the culture was shaken at 30°C until an OD_{600} of 1.0 was reached. Then, 1 mM IPTG was added to the culture and shaking was continued for 3 h. The cells were harvested and suspended in 10 ml of MTPBS [150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3 (*35*)] supplemented with 1% Triton X-100, 1 mM PMSF, and 1 μg each of leupeptin, pepstatin, and aprotinin per ml. The cells were lysed by sonication and then centrifuged at 8,000$\times g$ for 10 min at 0°C. The supernatant was transferred into a fresh tube and 100 μl of Glutathione Sepharose beads (Pharmacia) in MTPBS was added. The mixture was incubated overnight at 4°C with gentle shaking. The beads were then collected by centrifugation at 2,000$\times g$ for 5 min at 4°C, washed three times in MTPBS, and suspended in 100 μl of MTPBS with 0.01% sodium azide. Amounts of GST-fusion protein in the samples were determined using the protein assay kit as described above. To prepare T7-TBP, T7-TFIIB, and TFIIEβ, *E. coli* BL21(DE3) harboring plasmid pLysE (*24*) was introduced with either pET-TBP, pET-TFIIB, or pET-TFIIEβ. An ampicillin and chloramphenicol-resistant colony was isolated and grown to an OD_{600} of 1.0 at 30°C in 10 ml of LB broth supplemented with 50 μg of ampicillin and 20 μg of chloramphenicol per ml. IPTG was then added to a final concentration of 0.1 mM and shaking was continued for 3 h. The cells were harvested by centrifugation at 4°C, suspended in 1 ml of TGD_{100} buffer [20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM glutamate, 1 mM DTT (*36*)] supplemented with 1 mM PMSF and 1 μg each of aprotinin,

leupeptin, and pepstatin per ml, and lysed by sonication. The supernatant was recovered by centrifugation of the cell lysate at 0°C for 20 min at 14,000 rpm in a microcentrifuge.

Protein Binding Assays—Ten microliters of Glutathione Sepharose beads containing 20 μg of GST, GST-Pho2p, or GST-Pho4p was added to 100 μl of TGD_{100} buffer. Then, either 1 μg of purified T7-Pho4p sample (for GST and GST-Pho2p) or 5 μg of purified T7-Pho2p-His sample (for GST and GST-Pho4p) was added and the mixtures were incubated at 25°C for 1 h. The beads were collected by centrifugation at 14,000 rpm for 1 min at 4°C in a microcentrifuge, then washed five times with TGD_{100} buffer, and 10 μl of SDS-PAGE gel loading buffer was added. The mixture was boiled for 5 min, and SDS-PAGE and Western blotting were performed to detect bound proteins. For protein binding assays with T7-TBP, T7-TFIIB, and TFIIEβ, *E. coli* crude extracts containing 100 μg of total protein in 100 μl of TGD_{100} buffer were added to 10 μl of Glutathione Sepharose beads containing 20 μg of GST, GST-Pho2p, or GST-Pho4p. The mixture was subjected to protein binding assay, as described above.

RESULTS

Pho4p and Pho2p Form a Complex with DNA—Two-hybrid assays have suggested interaction between Pho2p and Pho4p (*14, 15*). This was also suggested by our genetic observations with temperature-sensitive *pho4* and *pho2* mutants. Briefly, mutants having single $pho2^{ts}$ or $pho4^{ts}$ mutation exhibited severely reduced acid phosphatase activities, *i.e.*, 1.4 milliunits/OD_{660} per ml in the $pho2^{ts}$ mutant and 2.5 milliunits in the $pho4^{ts}$ mutant, while the wild-type strain exhibited 452 milliunits at the restrictive temperature (35°C) in low-P_i medium. The same $pho2^{ts}$ and $pho4^{ts}$ mutants exhibited acid phosphatase activity of 133 and 19.3 milliunits, respectively, while the wild-type strain exhibited acid phosphatase activity of 383 milliunits, at the permissive temperature (25°C) in low-P_i medium. A double $pho4^{ts}$ $pho2^{ts}$ mutant with the same mutant alleles exhibited no detectable acid phosphatase activity in low-P_i medium even at 25°C. This phenomenon is similar to the synthetic lethal effect of two temperature-sensitive mutations in *Drosophila* and *S. cerevisiae* (*37*), and suggests that Pho2p and Pho4p form a complex or interact directly with each other.

To confirm complex formation of these proteins, the *PHO2-Myc* and *T7-PHO4* fusion genes were ligated into plasmid pYC209 marked with *LEU2* and YEp241 marked with *URA3*, respectively, then the plasmids were introduced into a *Δpho2 Δpho4* strain, NBD24-1A, and Ura^+ Leu^+ double transformants were isolated. Cells of these transformants were cultivated in high- or low-P_i medium and cell extracts were prepared. These cell extracts were incubated with the 36-bp fragment bearing the D5'A Pho4p binding site of *PHO84* (*8*) and subjected to immunoprecipitation with either anti-Pho4p or anti-Myc antibody. The precipitate was then subjected to Western blot analysis (Fig. 1). T7-Pho4p was detected in the immunoprecipitate with the anti-Myc antibody (Fig. 1, panel A2, lanes 1 and 2), and Pho2p-Myc was precipitated with the anti-Pho4p antibody (Fig. 1, panel B2, lanes 1 and 2). These results support the idea that Pho4p and Pho2p form a complex. However, no complex formation was detected in the

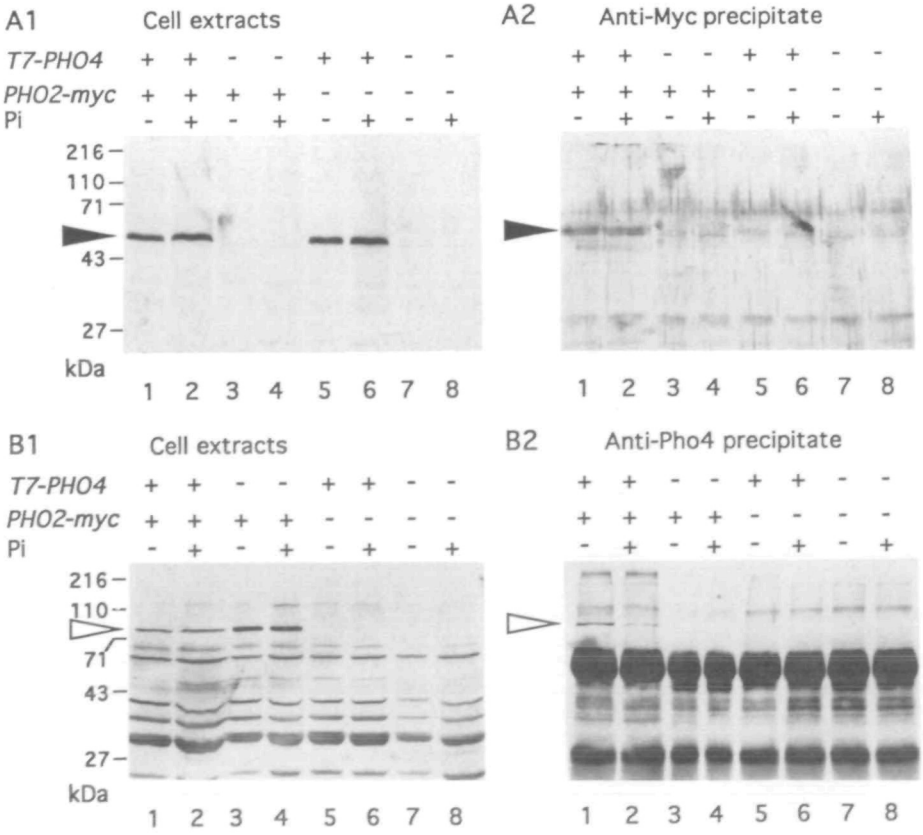

Fig. 1. **Co-immunoprecipitation of Pho2p and Pho4p.** Cell extracts were prepared from cells of NBD24-1A (*Δpho2 Δpho4*) harboring the following plasmid combinations: pYS143 (*T7-PHO4*) and pPHO2-Myc (*PHO2-Myc*) (lanes 1 and 2); YEp241 (T7-vector) and pPHO2-Myc (lanes 3 and 4); pYS143 and pYC209 (*myc*-vector; lanes 5 and 6); and pYC209 and YEp241 (lanes 7 and 8) cultivated in low-P_i(−) or high-P_i(+) medium and co-immunoprecipitation was performed as described in "MATERIALS AND METHODS." (A1) Detection of T7-Pho4p in crude extracts with anti-T7 monoclonal antibody. Cell extracts each containing 20 μg of protein were loaded in each lane. (A2) Detection of T7-Pho4p using the anti-Pho4p antibody after immunoprecipitation with the anti-Myc monoclonal antibody. (B1) Detection of Pho2p-Myc in crude extracts with anti-Myc monoclonal antibody. (B2) Detection of Pho2p-Myc using anti-Myc monoclonal antibody after immunoprecipitation with anti-Pho4p antibody. The closed arrowheads indicate the position of the T7-Pho4p, while the open arrowheads indicate the position of Pho2p-Myc. The numerals in the left margin represent the positions of the indicated molecular size markers.

absence of the 36-bp DNA (data not shown), as in the case of complex formation between Swi5p and Pho2p (*13*). This suggests that the interaction between these two proteins is weak *in vivo* and is enhanced with the DNA. The co-immunoprecipitation band of T7-Pho4p in the cell extract grown in high-P_i was similar in intensity to that observed at low P_i (Fig. 1, panel A2, lanes 1 and 2), while the band observed for Pho2p-Myc at high P_i was weaker than that at low P_i (Fig. 1, panel B2, lanes 1 and 2). Similar experiments to those of Fig. 1, panel B2, however, showed no significant difference between them (data not shown). Therefore, we favor the conclusion that complex formation between Pho2p and Pho4p is P_i-independent.

Pho4p and Pho2p Bind Directly—The above results indicated that Pho2p and Pho4p form a complex or interact with each other *in vivo*. To see if direct interaction between Pho2p and Pho4p can occur, we introduced plasmid pGST-PHO4 or pGST-PHO2 into *E. coli* DH5α. Cell extracts were prepared from the Amp^R transformants and the GST and GST-fusion proteins were adsorbed onto Glutathione Sepharose beads. Then, protein binding assays were performed using purified T7-Pho4p and T7-Pho2p-His samples and Western blot analysis was done to detect Pho4p-Pho2p binding. The results showed specific binding between GST-Pho4p and T7-Pho2p-His, while no binding was observed between GST and T7-Pho2p-His (Fig. 2, lanes 1 to 3). Similarly, GST-Pho2p could bind with T7-Pho4p (Fig. 2, lanes 4 to 6). These results indicate direct interaction between Pho2p and Pho4p even without the addition of DNA to the reaction mixture, while no Pho2p-Pho4p binding was detected in the co-immunoprecipitation experiments without UAS DNA, as described above. This discrepancy might be due to higher amounts of Pho2p and Pho4p present in the reaction mixture in the protein binding experiments, which were performed with purified preparations of proteins, than that in the co-immunoprecipitation experiments, which were done with crude yeast cell extracts. This also suggests that the binding affinity between Pho2p and Pho4p without DNA is weaker than that with DNA. The ladder pattern observed in lane 3 (Fig. 2) suggests easy degradation of T7-Pho2p-His.

Pho2p Enhances the Binding Affinity of Pho4p to DNA—Formation of a ternary complex was detected when Pho2p and Swi5p were combined with the *HO* promoter in a gel-retardation assay (*13*). Recently, similar ternary complex formation among Pho2p, Pho4p, and the *PHO5* promoter DNA has been demonstrated by DNase I footprinting and gel-retardation assays (*16*). To examine the effect of interaction between Pho4p and Pho2p *in vivo*, we performed DNA-binding experiments with yeast cell extracts. The *Δpho4* (NBD4-4A), *Δpho4 Δpho80* (NBD480-6C), and *Δpho4 Δpho2* (NBD24-1A) strains harboring the plasmid producing T7-Pho4p were cultivated in high- or low-P_i medium. Cell extracts were prepared and used to examine T7-Pho4p binding to a ^{32}P-labeled 36-bp fragment bearing UASp2, having a 5′-tcgacCCTTGGCACTC**ACACGTGGG**-

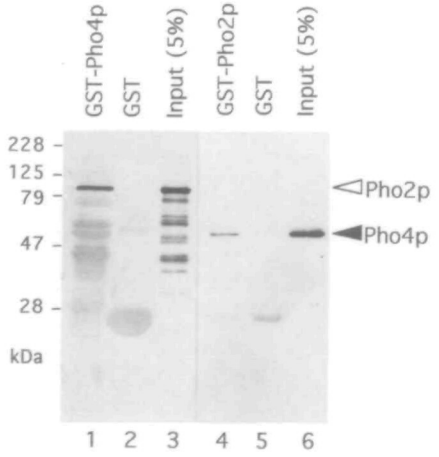

Fig. 2. **Pho4p and Pho2p interact directly.** Binding of T7-Pho2p-His with GST-Pho4p and that of T7-Pho4p with GST-Pho2p were examined by protein binding assays as described in "MATERIALS AND METHODS." Binding of T7-Pho2p-His to GST-Pho4p (lane 1) or to GST (lane 2) and that of T7-Pho4p to GST-Pho2p (lane 4) or GST (lane 5) were detected by Western blotting using anti-T7 monoclonal antibody. As controls in the binding experiments, 5% of the total amount of purified T7-Pho2p-His and T7-Pho4p samples used in the binding reaction was loaded into lanes 3 and 6, respectively. The arrows labeled Pho2p and Pho4p at the right margin represent the migration positions of T7-Pho2p-His and T7-Pho4p. The numerals at the left margin represent the migration positions of the molecular size markers.

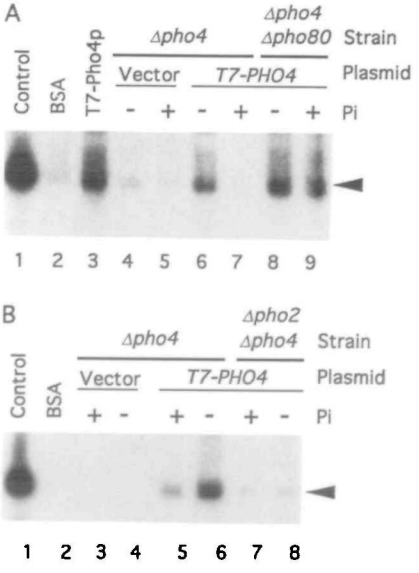

Fig. 3. **Pho2p enhances Pho4p binding to UASp2 *in vivo*.** (A) T7-Pho4p binding in *Δpho4* and *Δpho4 Δpho80* strains. Crude extracts containing 1.5 mg of protein from the transformants, NBD4-4A (*Δpho4*) [YEp131] (vector alone; lanes 4 and 5), NBD4-4A (*Δpho4*) [pYS104] (*T7-PHO4*; lanes 6 and 7), and NBD480-6C (*Δpho4 Δpho80*) [pYS104] (*T7-PHO4*) cultivated in low-P_i(−) or high-P_i(+) medium were mixed with 0.1 pmol of a ^{32}P-labeled 36-bp fragment bearing UASp2 of *PHO5* in a 200 μl reaction mixture and subjected to immunoprecipitation as described in "MATERIALS AND METHODS." Lane 1 was loaded with 1/50 amount of ^{32}P-labeled UASp2 fragment used in the binding reaction. One hundred micrograms of bovine serum albumin (BSA: lane 2) or 1 μg of T7-Pho4p (lane 3) was used instead of the crude yeast extract. (B) Binding of Pho4p in the *Δpho4* and *Δpho2 Δpho4* strains. Pho4p binding to UASp2 with the yeast extracts was performed as described in "MATERIALS AND METHODS." Lane 1, 1/50 amount of the ^{32}P-labeled UASp2 fragment used in the binding reaction; lane 2, 100 μg of BSA; lanes 3 and 4, NBD4-4A (*Δpho4*) [YEp131] (vector alone); lanes 5 and 6, NBD4-4A (*Δpho4*) [pYS104] (*T7-PHO4*); lanes 7 and 8, NBD24-1A (*Δpho4 Δpho2*) [pYS104] (*T7-PHO4*).

ACTAGCACAGg-3′ sequence covering the 30-bp region of the *PHO5* promoter (*38*) from nucleotide positions −265 to −236 [the uppercase letters indicate the 30-bp UASp2 region (*39*), the boldface letters in the center indicate the 9-bp *PHO* UAS motif (*8*), and the 6-bp lowercase letters indicate the *Sal*I cohesive ends], by immunoprecipitation of T7-Pho4p from the cell extracts by the addition of anti-T7 antibody (Fig. 3). This 36-bp UASp2 fragment of *PHO5* showed no appreciable binding of T7-Pho2p-His *in vitro*, due to the absence of any appreciable A/T sequence in the fragment (to be published elsewhere). It was confirmed that the amounts of T7-Pho4p in the cell extracts were not affected by P_i concentration in the medium. However, stronger binding of T7-Pho4p to the ^{32}P-labeled 36-bp UASp2 fragment was observed in the precipitate from *PHO2*$^+$ cells harboring *T7-PHO4* plasmid cultivated at low P_i than in that prepared from cells cultivated in high-P_i medium (Fig. 3A, lanes 6 and 7; Fig. 3B, lanes 5 and 6). The observed difference might be due to P_i signals, because no difference in binding signals was observed with the cell extracts prepared in high- and low-P_i media in a *Δpho80* mutant (Fig. 3A, lanes 8 and 9). The binding of Pho4p to the UASp2 fragment was significantly decreased in the *Δpho2* cells (Fig. 3B, lanes 7 and 8), while no appreciable binding of Pho2p to the UASp2 fragment was observed, as described above. These results strongly suggests that interaction between Pho2p and Pho4p enhances the binding affinity of Pho4p or the Pho4p-Pho2p complex to the UAS site under low-P_i conditions.

The *in vitro* assays by Barbarić *et al.* (*16*) indicated that ternary complex formation of Pho4p and Pho2p on the *PHO5* promoter required the presence of the binding sites of both Pho4p and Pho2p. However, we could detect Pho4p-Pho2p interaction in the protein binding assay without DNA (Fig. 2) and experiments using crude yeast extracts (Fig. 3) showed that Pho2p enhances Pho4p binding affinity to the 36-bp UASp2 fragment of *PHO5* even in the absence of a Pho2p-binding site, as mentioned above. These results suggest that interaction of Pho2p induces conformational change in Pho4p, allowing it to bind to the UAS sequence with increased affinity, as suggested by Shao *et al.* (*15*).

Pho4p and Pho2p Interact with General Transcription Factors—Transcriptional regulators bound to an enhancer region in a promoter may affect the initiation of transcription by interacting with at least two of the general transcription factors, TFIID and TFIIB (*20, 40*). Interaction of the Pho4p activation domain with the transcription machinery has been suggested by the two-hybrid assay (*15*). To see if Pho4p and/or Pho2p are able to interact directly with some of the components of the transcriptional machinery, T7-fusion genes of TBP, TFIIB, and TFIIEβ, were constructed and expressed in *E. coli* as described in "MATERIALS AND METHODS." The cell extracts were prepared and subjected to protein binding assay with GST, GST-Pho2p, and GST-Pho4p bound to Glutathione Sepharose beads and the binding of these proteins to the transcription factors was detected by Western blot analysis. We found that GST-Pho2p and GST-Pho4p showed specific binding with TBP, while no binding was observed with GST alone (Fig. 4, lanes 1 to 3). Similar results were also observed when protein binding assays were performed with T7-TFIIB (Fig. 4, lanes 5 to 8) and TFIIEβ (lanes 9 to 12). These

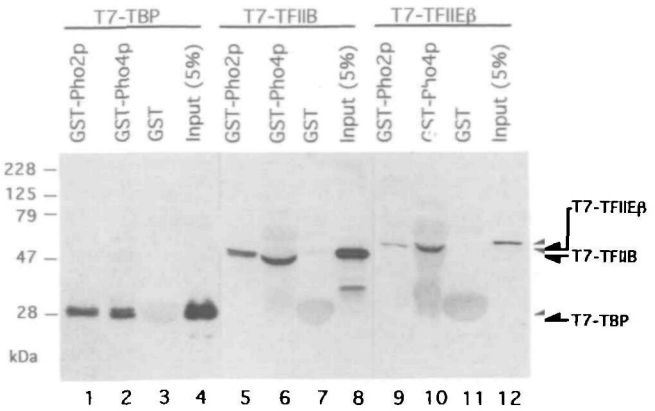

Fig. 4. **Pho4p and Pho2p can bind with TBP, TFIIB, and TFIIEβ.** Binding of T7-TBP, T7-TFIIB, and T7-TFIIEβ with GST-Pho4p and GST-Pho2p was examined by means of protein binding assays as described in "MATERIALS AND METHODS." Binding of T7-TBP, T7-TFIIB, and TFIIEβ to GST-Pho2p (lanes 1, 5, and 9, respectively), to GST-Pho4p (lanes 2, 6, and 10, respectively) and to GST (lanes 3, 7, and 11, respectively) were detected by Western blotting using anti-T7 monoclonal antibody. As controls, the indicated amounts of crude *E. coli* extract containing the T7-fusion proteins used in the binding reaction were loaded into lanes 4, 8, and 12, respectively. The arrows labeled T7-TBP, T7-TFIIB, and T7-TFIIEβ at the right margin represent the migration positions of the indicated proteins. The numerals on the left margin represent the migration positions of the molecular size markers.

results strongly suggest that Pho4p and Pho2p bind or interact directly with the above general transcription factors *in vivo*. It was noted that GST-Pho4p has somewhat higher affinity to these transcription factors than GST-Pho2p.

DISCUSSION

Pho2p (*i.e.*, Bas2p and Grf10p) is a general regulatory factor and influences the transcription of several genes in *S. cerevisiae*. It was demonstrated that Swi5p and Pho2p interact cooperatively on the *HO* promoter (*13*). Recent *in vitro* experiments have demonstrated that Pho4p and Pho2p bound cooperatively on the *PHO5* promoter (*16*). Our observations with *ts*-mutants and the results of DNA-binding experiments also indicate that Pho2p enhances the transcriptional activity of Pho4p by increasing its ability to bind the UAS. Thus, Pho4p and Swi5p might activate their target genes through similar mechanisms. Swi5p is localized in the cytoplasm at the G2 phase of the cell cycle due to its phosphorylation by the cyclin-Cdc28 protein kinase; it enters the nucleus at Start in the G1 phase and activates *HO* expression (*41*). Similarly, Pho4p is localized in the cytoplasm under repressed conditions (*4*) due to hyperphosphorylation of it by the Pho80p-Pho85p cyclin-CDK complex (*3*). The results of our preliminary study indicated that the hyperphosphorylation of Pho4p did not affect its ability to bind to DNA (unpublished results). Upon entry into the nucleus, both Swi5p (*42*) and Pho4p interact with Pho2p and enhance transcription of their target genes. No significant homology, however, was detected between the aa sequences of Pho4p and Swi5p.

Another bHLH protein, Cpf1p, binds to the 5′-CACGTG-3′ motif, and was shown to bind to Pho4p binding sites both

in vitro (*43*) and *in vivo* (*44*). Genetic data also suggest that Pho4p can affect the expression of some genes targeted by Cpf1p (*45*) indicating that the DNA binding specificity of Pho4p and Cpf1p is low. Thus, we speculate that the binding specificity of Pho4p towards the *PHO* UASs is enhanced by interaction with Pho2p as shown in Fig. 3. A similar mechanism has been suggested for the discrimination of Swi5p and Ace2p in their binding to the *HO* promoter (*13*). Shao *et al.* (*15*) recently suggested that interaction with Pho2p increases the accessibility of the activation domain of Pho4p to the transcriptional machinery. Thus, the interaction of Pho4p and Pho2p may result in two major effects; first, it enables Pho4p to bind its target sites properly; and second, it increases the accessibility of the Pho4p activation domain, thereby enhancing its transcriptional activity. The findings that binding of Pho4p to DNA alone is not sufficient to bring about nucleosome disruption in the *PHO5* promoter, an event that accompanies derepression (*44, 46*), and that no nucleosome disruption of the *PHO5* promoter was observed in a *pho2* mutant (*44*) support these possibilities.

Eukaryotic transcriptional activators stimulate RNA polymerase II activity by enhancing the formation of a pre-initiation complex through contacts with one or more components of the basal transcription machinery (*19*). Interactions between TBP and the transcriptional activators VP16 (*20*), Zta (*47*), E1A (*48*), and Tat (*49*) have been reported. Pho4p, like VP16, also bears an acidic transactivation domain and we have shown by protein binding assay (Fig. 4, lane 2) that Pho4p can also interact with TBP. TFIIB, whose recruitment to the pre-initiation complex is enhanced by transcriptional regulators (*19, 40*), also showed specific contact or binding with Pho4p by protein binding assay (Fig. 4, lane 6). Likewise, interaction of Pho4p and TFIIEβ, a component of TFIIE, was also observed (Fig. 4, lane 10). Unlike Pho4p, Pho2p does not have a known transcriptional activation domain. The results of protein binding assays revealed that it also has affinity to TBP, TFIIB, and TFIIEβ, though somewhat weaker than that of Pho4p. The ability of Pho2p to interact with the basal transcription factors is not very surprising, since it functions as a general regulator with broad specificity for various genes. Therefore, it can be imagined that a Pho4p-Pho2p complex bound to the *PHO* promoters can stimulate transcription of the *PHO* genes through direct contacts with transcription factors.

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