The transcription factor, the Cdk, its cyclin and their regulator: directing the transcriptional response to a nutritional signal

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The Pho80–Pho85 cyclin–cdk complex prevents transcription of *PHO5* by inhibiting the ability of the basic-helix–loop–helix transcription factor Pho4 to activate transcription in response to high phosphate conditions. In low phosphate the Pho80–Pho85 complex is inactivated and Pho4 is then able to activate the acid phosphatase gene *PHO5*. We show here that Pho4 and the homeobox protein Pho2 interact *in vivo* and act cooperatively to activate the *PHO5* UAS, with interaction being regulated by the phosphate switch. In addition, we also demonstrate that an additional factor, Pho81, interacts in high phosphate with both the Pho80 cyclin and with Pho4. In low phosphate, Pho80 and Pho81 dissociate from Pho4, but retain the ability to interact with each other. The evidence presented here supports the idea that Pho81 acts as a phosphate-sensitive trigger that regulates the ability of the Pho80–Pho85 cyclin–cdk complex to bind Pho4, while DNA binding by Pho4 is dependent on the phosphate-sensitive interaction with Pho2.

Key words: cyclin–cdk inhibitor/PHO5/PHO81/phosphate switch/transcription regulation

Introduction

A major checkpoint in the control of the eukaryotic cell cycle, comprising the sequential passage through G_1, S, G_2 and M phases, is an event termed START, occurring late in G_1, after which a cell is committed to undergoing a further round of cell division. Before START the cell may exit the cell cycle by undergoing arrest in G_1, an event that may be triggered by a number of environmental cues, most notably the presence of mating pheromones and the limitation of essential nutrients. The cyclin-dependent kinases (cdks), particularly Cdc28/Cdc2, and their regulatory subunits, the cyclins, have been implicated as crucial elements in control of the cell cycle (for reviews see Nurse, 1990; Reed, 1992; Nasmyth, 1993). In yeast, the activity of the Cdc28 kinase is determined by its association with a variety of different cyclins, which may be expressed at specific stages of the cell cycle and which target Cdc28 to its various substrates. In mammalian cells, progress through the cell cycle is characterized by specific combinations of cdks and their associated cyclins, with each combination presumably performing specific functions (van den Heuvel and Harlow, 1993).

While the differential interaction between the cyclins and the cdks is a major determinant in regulating the cell cycle, an additional level of control is exerted by proteins, such as Far1 (Peter *et al.*, 1993) or members of the p21 family (Gu *et al.*, 1993; Gyuris *et al.*, 1993; Harper *et al.*, 1993; Serrano *et al.*, 1993; Xiong *et al.*, 1993; Polyak *et al.*, 1994), which act to inhibit cdk function. These cdk-inhibitory proteins are likely to play a crucial role in integrating the action of the cyclin–cdk complexes with various extracellular and intracellular cues. How such signals govern the complex interplay between the various components of the cell cycle machinery is a key issue.

An opportunity to dissect a regulatory pathway leading from an intracellular signal via a cyclin–cdk complex to a transcriptional response is provided by the yeast PHO regulon, which responds to intracellular levels of an essential nutrient, phosphate. When the supply of inorganic phosphate becomes limiting, yeast react by coordinately inducing a set of phosphatase genes, most notably *PHO5* whose product acts as an extracellular phosphate scavenger. Under high phosphate conditions, the expression of *PHO5* is repressed (Kramer and Andersen, 1980; Lemire *et al.*, 1985). Several genes have been identified by genetic analysis to be essential for the regulation of the *PHO5* gene (Toh-e *et al.*, 1973; Toh-e and Oshima, 1974; Ueda *et al.*, 1975; Yoshida *et al.*, 1989; Gilliquet *et al.*, 1990; Madden *et al.*, 1990; Vogel and Hinnen, 1990; Bun *et al.*, 1991). The strong induction of *PHO5* transcription under conditions of phosphate starvation is mediated by the action of basic-helix–loop–helix (bHLH) transcription factor Pho4 and the homeobox protein Pho2 (Bas2/Grf10) (Burglin, 1988; Vogel *et al.*, 1989; Yoshida *et al.*, 1989; Berben *et al.*, 1990; Ogawa and Oshima, 1990; Fisher *et al.*, 1991; Fascher *et al.*, 1993). While Pho4 is the major activator, the Pho2 protein plays an essential but uncharacterized role (Fascher *et al.*, 1990). In high phosphate, repressing conditions, the ability of Pho4 to activate transcription is blocked by the products of the *PHO80* and *PHO85* genes (Uesono *et al.*, 1987; Madden *et al.*, 1988; Gilliquet *et al.*, 1990). Pho80 is a cyclin (Kaffman *et al.*, 1994) sharing significant homology with the cyclins Hcs26 (Ogas *et al.*, 1991) and OrfD (Fröhlich *et al.*, 1991), and is required for phosphorylation of Pho4 by Pho85 (Kaffman *et al.*, 1994), a member of the p34$^{Cdc2/Cdc28}$-related kinase family (Toh-e *et al.*, 1988). However, how phosphorylation of Pho4 inhibits its ability to activate transcription is not known.

For derepression/activation of *PHO5* in low phosphate conditions an additional, 134 kDa, protein, Pho81, is required (Coche *et al.*, 1990; Creasy *et al.*, 1993; Ogawa *et al.*, 1993). How Pho81 functions is also unknown, but the observation that Pho81 contains six ankyrin repeats

(Ogawa et al., 1993) suggests that it may participate in protein–protein interactions.

In this report, we characterize the interactions between the regulatory components of the PHO regulon in response to the phosphate switch. The results obtained are consistent with a model in which, under high phosphate conditions, Pho4 is repressed by the Pho80–Pho85 cyclin–cdk complex which can mask the Pho4 activation domain, while DNA binding by Pho4 is blocked by the inhibition of its ability to interact with the homeobox factor Pho2. In high phosphate, in addition to interacting with the Pho85 cdk and Pho4, the Pho80 cyclin also interacts with Pho81. In low phosphate, Pho81 and Pho80 dissociate from Pho4 but remain associated with each other, while Pho4 is allowed to bind co-operatively to the PHO5 UAS with Pho2. The evidence presented here supports the idea that Pho81 acts as a phosphate-sensitive trigger that regulates the ability of the Pho80–Pho85 cyclin–cdk complex to bind Pho4 and regulate its ability to activate transcription.

Results

The phosphate switch regulates the interaction between Pho4 and Pho2 in vivo

While it is clear that the Pho80–Pho85 cyclin–cdk complex represses the ability of Pho4 to activate transcription, how repression is accomplished remains unresolved. Two mechanisms to account for repression of Pho4 by the Pho80–Pho85 cyclin–cdk complex may be envisaged; inhibition of DNA binding, and inactivation of the PHO4 activation domain. To determine whether the ability of Pho4 to bind DNA is regulated by the phosphate switch, we expressed a Pho4-VP16 chimera under high and low phosphate conditions in a yeast strain, Y704, in which the endogenous PHO4 gene is disrupted; the potent VP16 activation domain is not subject to repression by Pho80 (Jayaraman et al., 1994) and if DNA binding by Pho4 were not regulated, the Pho4-VP16 chimera would be able to activate transcription irrespective of phosphate concentration. In contrast, the results (Figure 1) demonstrate that the Pho4-VP16 chimera activates transcription efficiently only under conditions of phosphate starvation, suggesting that the phosphate switch may regulate PHO4 DNA binding.

Two binding sites for Pho4, P1 and P2, are present in the PHO5 UAS. Under high phosphate, repressing conditions the low affinity P1 site is present in a DNase I-hypersensitive site while the Pho2 binding site and the high affinity P2 element are located within an adjacent positioned nucleosome (Almer and Horz, 1986; Almer et al., 1986). Derepression under low phosphate conditions is accompanied by the selective removal of four nucleosomes across this region (Almer and Horz, 1986). While Pho4 is the primary trigger for transcription activation and nucleosome disruption, the Pho2 protein plays an essential but uncharacterized role (Fascher et al., 1990).

To account for our results and at the same time provide a role for Pho2, we conceived a model in which Pho2 would interact in a phosphate-regulated fashion with Pho4 to facilitate binding to the weak P1 site in the PHO5 UAS. To test this model and to assay directly for Pho4–Pho2 interaction, we made use of a two-hybrid system in which Pho2 was expressed as a fusion with the bacterial LexA
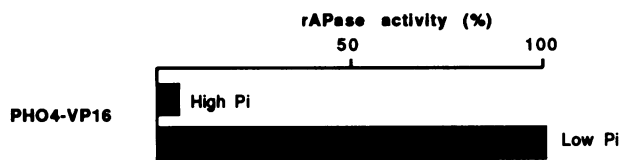

Fig. 1. Transcription activation by a Pho4-VP16 chimeric protein is sensitive to the phosphate switch. Yeast strain Y704 (pho4:HIS3) was transformed with a vector expressing Pho4-VP16 from the constitutive PHO4 promoter on a low copy number (LCN) vector and yeast grown under high (7.4 mM) or low (3.7 μM) phosphate conditions assayed for repressible acid phosphatase (rAPase) activity using whole cells as a source of enzyme and p-nitrophenol phosphate (Sigma 104) as substrate.

repressor while Pho4 was expressed with or without a VP16-tag (Figure 2A). The results obtained (Figure 2B) demonstrate that both Pho4 and Pho4-VP16 can interact with Pho2, resulting in activation up to 60-fold greater than that seen using the LexA-Pho2 chimera alone. The ability of these proteins to interact and thereby activate transcription from a Lex operator-CYC-lacZ reporter was also assayed under both high and low phosphate conditions. Consistent with the Pho4–Pho2 interaction being regulated by the phosphate switch, interaction between LexA-Pho2 and Pho4-VP16 was only detected under low phosphate conditions (Figure 2C).

To map the region of Pho4 involved in interaction with Pho2 we expressed a series of VP16-tagged Pho4 deletion mutants. The results (Figure 2D) demonstrate that deletion mutants lacking N-terminal residues up to amino acid 156 (ΔN156) interact with Pho2 and thereby activate transcription efficiently. Deletion ΔN200, although activating ~4-fold less well than the WT Pho4-VP16 protein, nevertheless activates ~16-fold better than the LexA-Pho2 chimera alone. In contrast, removal of a further 18 amino acids (ΔN218) abolishes interaction with Pho2. The internal deletion mutants (Δ94int, Δ156int and Δ200int), also fail to interact, with the Δ200int mutant lacking only amino acids 200–247. Since all the Pho4-VP16 mutants that fail to interact with Pho2 are expressed in yeast and can interact with Pho80 (Jayaraman et al., 1994), we conclude that sequences between residues 200 and 218 are essential for interaction with Pho2.

To ask whether this region is sufficient to mediate interaction with Pho2, Pho4 residues between amino acids 190 and 230 were fused in-frame to LexA and cotransformed into yeast together with a vector expressing a VP16-Pho2 fusion protein. In agreement with the previous data, these 40 amino acids derived from Pho4 enabled the VP16-Pho2 chimera to activate transcription from the Lex operator-CYC-lacZ reporter (Figure 2E) and are therefore sufficient to mediate interaction in this assay.

The results from the two-hybrid system demonstrated clearly that Pho4 and Pho2 can interact in vivo and that this interaction was regulated by the phosphate switch. Under physiological conditions, Pho2 is essential for activation of the PHO5 UAS by Pho4 (see Fascher et al., 1990 for example) and a mutant Pho4 protein tagged with the VP16 activation domain but lacking sequences required for interaction with Pho2 fails to activate the PHO5 UAS under high or low phosphate conditions (our unpublished observations). Taken together therefore, the evidence sug-
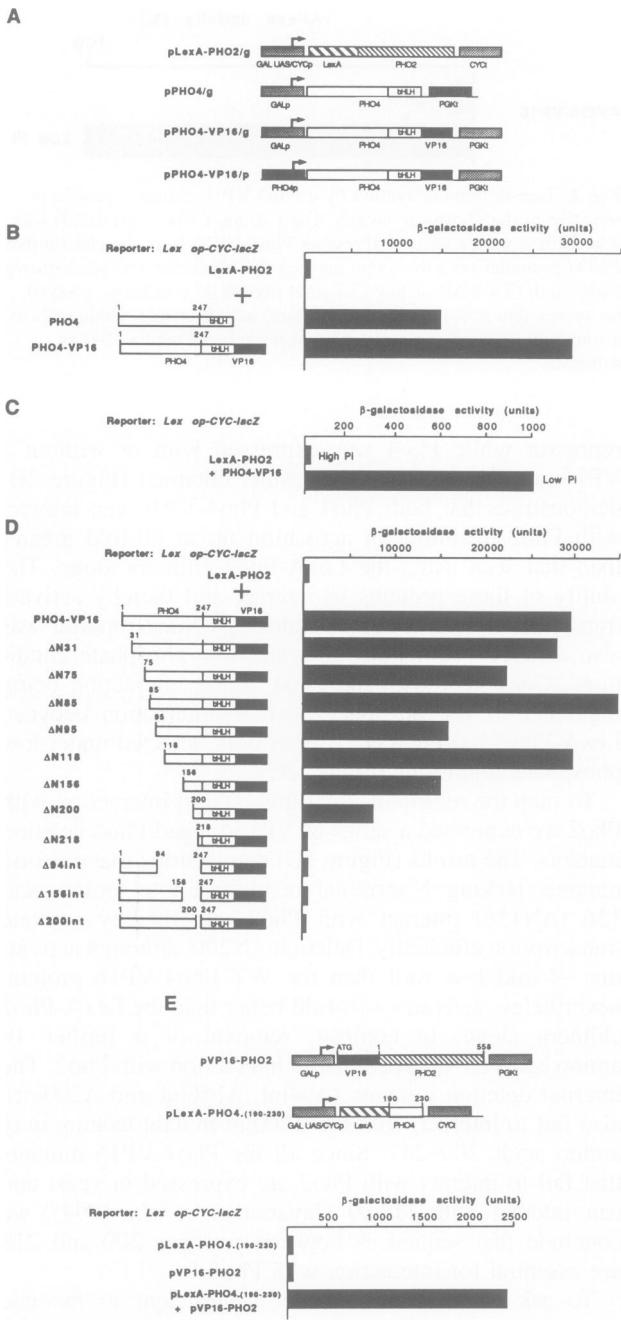
Fig. 2. Interaction between Pho4 and Pho2 in a two-hybrid assay is phosphate sensitive and requires a region of Pho4 located between amino acids 200 and 218. (A) The expression vectors used. LexA-Pho2, Pho4 or Pho4-VP16 proteins were expressed from either the galactose-inducible *GAL10* or *GAL/CYC* promoters, or from the constitutive *PHO4* promoter as indicated. (B) Pho4 and Pho2 interact in a two-hybrid assay. Yeast strain Y704 *(pho4:HIS3)* was transformed with plasmid pLexA-PHO2/g alone or together with either pPHO4/g or pPHO4-VP16/g and the level of β-galactosidase activity derived from a Lex operator-*CYC-lacZ* reporter determined after growth in galactose minimal medium. (C) The interaction between Pho4 and Pho2 is regulated by phosphate levels. Yeast were transformed with pLexA-PHO2/g and pPHO4-VP16/p and grown in high (7.4 mM) or low (3.7 μM) phosphate glucose minimal medium and the level of β-galactosidase activity obtained from the Lex operator-*CYC-lacZ* reporter measured. (D) A region of Pho4 between amino acids 200 and 218 is essential for interaction with Pho2. Yeast strain Y704 was transformed with pLexA-PHO2/g alone or together with pPHO4-VP16/g or derivatives expressing the indicated N-terminal or internal in-frame deletions and β-galactosidase activity derived from a Lex operator-*CYC-lacZ* reporter determined. Note that all Pho4 mutants are expressed efficiently *in vivo* (Jayaraman *et al.*, 1994). (E) Pho4 residues 190–230 are sufficient for interaction with Pho2. VP16-Pho2 or a LexA protein fused in-frame to Pho4 amino acids 190–230 were expressed either alone or together in strain Y704 and the activity of the LexA operator-*CYC-LacZ* reporter was determined.

gests strongly that Pho4 and Pho2 bind DNA cooperatively in a phosphate-sensitive fashion.

To investigate this possibility, we devised an *in vivo* cooperation assay for Pho4 and Pho2 DNA binding using the endogenous repressible acid phosphatase (rAPase) gene as a reporter. In this assay Pho2, which posesses only a weak activation domain, is expressed as a VP16 fusion either alone or together with a derivative of Pho4 deleted to amino acid 156 and lacking its entire activation domain (PHO4ΔN156). This mutant Pho4 protein contains all sequences required for DNA binding and retains the ability to interact with both the Pho80—Pho85 complex (Jayaraman *et al.*, 1994) and with Pho2 (see Figure 2D). The vectors used for expressing these proteins are shown in Figure 3A. If an interaction between Pho4 and Pho2 is essential for each protein to bind the *PHO5* UAS, then

we would predict that the VP16-Pho2 chimera would fail to activate transcription when expressed alone in a strain (Y704) lacking endogenous Pho4, but would do so if the transcriptionally inert Pho4ΔN156 mutant were present. Moreover, the results from the two-hybrid assay (Figure 2C) would also predict that any activation resulting from cooperative DNA binding *in vivo* between Pho4ΔN156 and VP16-Pho2 should be regulated by the phosphate switch. The results from such an experiment, which are most easily visualized by assaying repressible acid phosphatase (rAPase) activity *in situ*, are shown in Figure 3B.

Consistent with Pho2 being unable to bind the *PHO5* UAS in the absence of Pho4, the VP16-Pho2 protein fails to activate transcription of the rAPase gene under either high or low phosphate conditions (Figure 3B, row 2). As

A

B
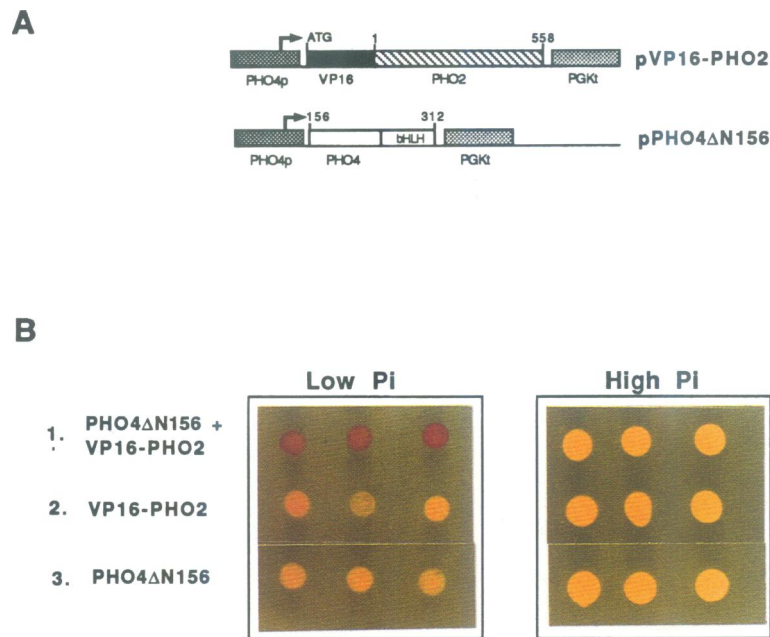
Fig. 3. Pho4 and Pho2 act co-operatively on the *PHO5* UAS. (**A**) The VP16-Pho2 and Pho4ΔN156 expression vectors. pVP16-Pho2 expresses the entire Pho2 protein as a C-terminal in-frame fusion with the VP16 activation domain. pPho4ΔN156 expressess a Pho4 derivative lacking residues N-terminal to amino acid 156 which renders the protein transcriptionally inert. Both plasmids are based on the low copy number (LCN) pRS vectors and contain the VP16-Pho2 or Pho4ΔN156 coding sequences under the control of the constitutive *PHO4* promoter with the *PGK* transcription terminator located 3′ to the expressed coding sequence. (**B**) *In situ* rAPase assays performed on the *pho4* disruption strain Y704 expressing the indicated proteins. Yeast were transformed with pVP16-PHO2 (row 2) or pPHO4ΔN156 (row 3) alone or co-transformed with both plasmids (row 1) and assayed for rAPase activity using an agar overlay and α-napthyl acid phosphate as substrate. rAPase activity results in formation of a red product against a yellow background (green after photographic processing!). No rAPase activity was detected under high phosphate conditions. The results from three independent transformants are shown for each combination of transforming plasmids. All the yeast colonies shown were taken from the same high or low phosphate plate and were assayed simultaneously under the same conditions.
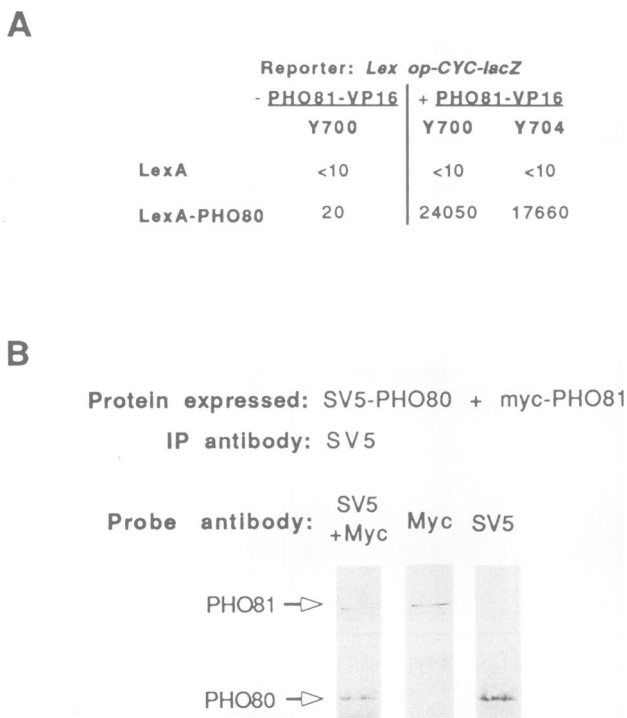
expected, the Pho4ΔN156 derivative is also transcripionally inert in both high and low phosphate (Figure 3B, row 3). Strikingly, however, co-expression of VP16-Pho2 with Pho4ΔN156 activates transcription of the endogenous rAPase gene (Figure 3B, row 1) in a phosphate-sensitive fashion. For VP16-Pho2 to activate transcription it must bind DNA, and since activation is only achieved in the presence of Pho4, these results strongly suggest that Pho2 and Pho4 interact to facilitate DNA binding.

Pho81 interacts with the Pho80 cyclin

While repression of Pho4 is mediated by the Pho80–Pho85 complex, an additional protein, Pho81, is essential for activation of the *PHO5* UAS by Pho4. Several possible mechanisms by which Pho81 might exert an effect can be envisaged. For example, Pho81 could interact with Pho80 or Pho85 and act to disrupt the interaction of the Pho80–Pho85 complex with Pho4. Alternatively, Pho81 might bind Pho4 in such a way as to counteract the effects of the cyclin–cdk repressor complex. In an attempt to distinguish between these models we fused Pho81 with the VP16 activation domain and asked first whether it could interact *in vivo* with LexA-Pho80. Expression of the Pho81-VP16 chimera alone failed to activate the Lex operator-*CYC-lacZ* reporter (Figure 4A). On the other hand, highly efficient activation was observed if Pho81-VP16 was co-expressed with LexA-Pho80, demonstrating that Pho80 and Pho81 interact *in vivo*. Since this experiment was performed in a strain, Y700, that expresses endogenous Pho4, we also repeated the experiment in a strain, Y704, in which the *PHO4* gene is disrupted, to

determine whether the Pho80–Pho81 interaction detected was Pho4-dependent. Using the Y704 strain a similar level of transcription activation mediated by interaction between the LexA-Pho80 and Pho81-VP16 proteins was evident, demonstrating that the interaction of Pho81 and the Pho80 cyclin did not require Pho4. We also asked whether the Pho81-VP16 protein could interact with a LexA-Pho85 chimera. In most experiments (not shown), no interaction between these two proteins was observed. In some cases, however, activation was seen in strains expressing endogenous Pho80 but was never observed in a strain containing a *pho80* disruption, suggesting that any interaction between Pho81 and Pho85 is indirect and requires Pho80.

To verify the results obtained from the two-hybrid system using a more biochemical approach, we next expressed Pho80 and Pho81 in yeast as epitope-tagged proteins and assayed for interaction using an initial immunoprecipitation followed by Western blotting using the appropriate anti-epitope antibodies. Figure 4B shows the results obtained when Pho81 tagged with the Myc 9E10 epitope is co-expressed with Pho80 tagged with the SV5 epitope. Extract from yeast expressing the two proteins was first immunoprecipitated using the SV5 antibody and the immunoprecipitate subsequently Western blotted and probed with either anti-SV5, anti-myc 9E10 or both antibodies. Using both the anti-SV5 and anti-Myc 9E10 antibodies as probes, bands corresponding to both Pho80 and Pho81 are visible. Using either antibody alone results in only one or other of the two proteins being visible, demonstrating the specificity of each probe antibody, while

A

Reporter: *Lex op-CYC-lacZ*

	- PHO81-VP16	+ PHO81-VP16	
	Y700	Y700	Y704
LexA	<10	<10	<10
LexA-PHO80	20	24050	17660

B

Protein expressed: SV5-PHO80 + myc-PHO81

IP antibody: SV5

Probe antibody: SV5 +Myc | Myc | SV5

PHO81 →▷

PHO80 →▷

Fig. 4. Interaction between Pho81 and Pho80 *in vivo*. (A) Yeast strains Y700 (*ade2, leu2, trp1, his3, ura3*) and Y704 (*ade2, leu2, trp1, his3, ura3, pho4:HIS3*) were transformed with vectors expressing LexA-Pho80 and Pho81-VP16 fusion proteins and β-galactosidase activity arising from activation of the Lex operator-*CYC-lacZ* reporter determined. (B) Co-immunoprecipitation of Pho80 and Pho81. Pho80 and Pho81were tagged at their N-termini with the SV5 or myc 9E10 epitopes respectively and expressed in yeast strain Y700. Extract from expressing yeast was immunoprecipitated with anti-SV5 antibody, the precipitate split into three aliquots, subjected to SDS–PAGE and Western blotted. The three samples were probed with either anti-SV5, anti-Myc 9E10 or both antibodies as indicated. Control experiments (not shown) verified that the SV5 antibody could not immunoprecipitate the Myc-tagged Pho81 protein.

additional control experiments (not shown) confirmed that the SV5 antibody did not react with the Myc-tagged Pho81 protein. Similar results were also obtained using a strain, Y704, in which the *PHO4* gene is disrupted (data not shown), again demonstrating that Pho4 is not required for formation of the complex between the Pho80 cyclin and its regulator Pho81. The complex between these two proteins can therefore be detected using both co-immuno-precipitation and the two-hybrid assay.
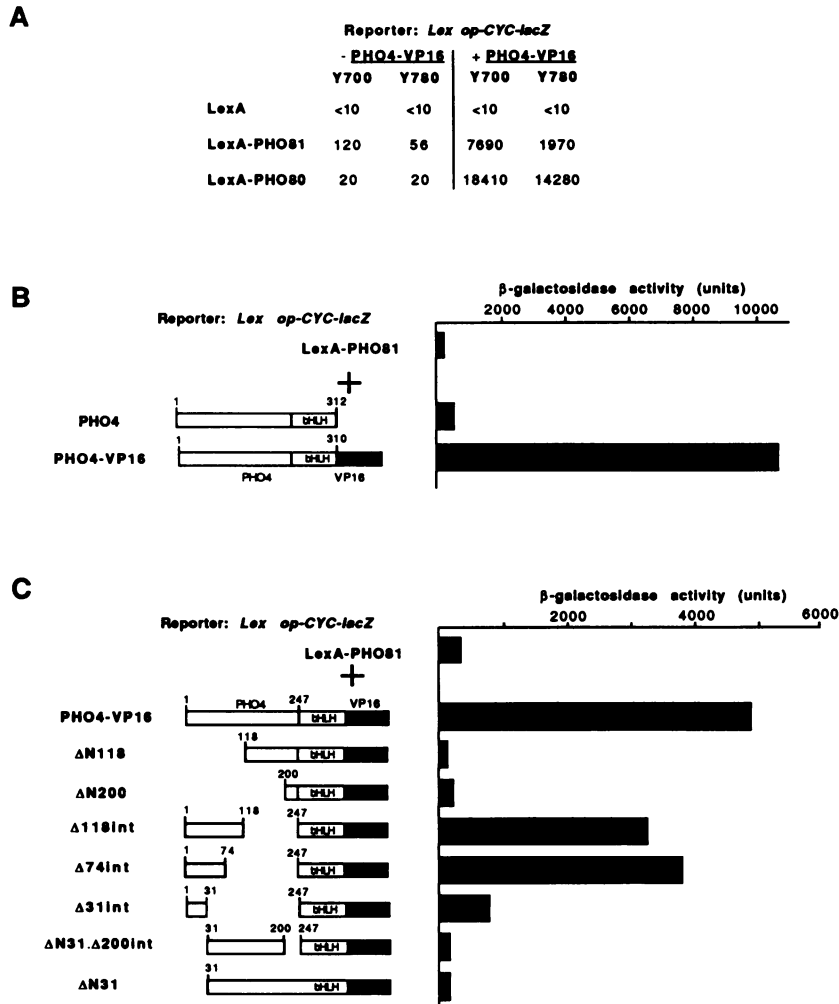
The Pho81 cyclin inhibitor also interacts with the Pho4 transcription factor

The data presented above demonstrate that Pho81 interacts with the Pho80 cyclin and raised the possibility that derepression of Pho4 under low-phosphate conditions was a direct result of Pho81 disrupting the Pho4−Pho80 complex through interaction with the Pho80 cyclin. Since the interaction between Pho80 and Pho81 did not require Pho4 it seemed unlikely that Pho81 would interact with Pho4 directly. Nevertheless, to eliminate this possibility, a LexA-Pho81 chimera was co-expressed with the Pho4-VP16 protein and their ability to activate transcription from the Lex operator-*CYC-lacZ* reporter determined. Contrary to expectations, efficient activation, >60-fold

greater than that obtained by expression of LexA-Pho81 in the absence of Pho4-VP16, was observed when both proteins were co-expressed (Figure 5A), demonstrating that while Pho81 could interact with Pho80, it could also bind Pho4. Although it was evident that Pho4 could bind Pho81, there remained a possibility that interaction with Pho4 was not a direct event, but was mediated by Pho80−Pho4 complex formation. To determine whether Pho80 was required for the interaction of LexA-Pho81 with Pho4-VP16, the two proteins were co-expressed in a strain, Y780, which contains a *pho80* disruption. The levels of transcription activation detected by co-expression of LexA-Pho81 and Pho4-VP16 were reproducibly ~3- to 4-fold less in the *pho80* disruption strain than in the strain expressing Pho80. Although it is difficult to make direct comparisons between results obtained in different strains, this 3-fold difference, which may be significant since similar levels of transcription activation in the two strains were obtained by co-expressing LexA-Pho80 with Pho4-VP16, may indicate that while Pho81 and Pho4 can interact in the absence of Pho80, Pho80 may contribute to the stability of the complex. Nevertheless, it is clear that irrespective of whether Pho80 does stabilize the interaction, Pho81 can bind Pho4 in the absence of Pho80.

While it is evident that the interaction between Pho80 and Pho81 detected using the two-hybrid assay can be reproduced faithfully using epitope-tagged proteins and immunoprecipitation/Western blotting, and we can detect readily the interaction between Pho4 and Pho81 using the two-hybrid system, we have to date been unable to co-immunoprecipitate epitope-tagged Pho4 and Pho81. While other explanations are possible, our failure to detect an interaction between Pho4 and Pho81 by co-immunopre-cipitation most likely indicates that the interaction between Pho81 and Pho4 is relatively unstable and does not survive the washing procedure involved in the co-immunoprecipi-tation assay. A similar difference between the results obtained in the two-hybrid assay and co-immunoprecipi-tation has been described for the interaction between RAS and RAF; an interaction between RAS and RAF, which is supported by functional studies using RAS mutants, can be detected readily using the two-hybrid assay but not by co-immunoprecipitation, leading to the conclusion that the two-hybrid assay may be particularly well suited to monitoring weak protein−protein interactions of biolo-gical significance (van Aelst *et al.*, 1993).

Although it was not possible to detect a Pho81−Pho4 complex using a co-immunoprecipitation assay, the results from the two-hybrid system raised the intriguing possibility that Pho81 could interact both with Pho4 and Pho80 and that Pho81 might play a dual role in activation by Pho4: on the one hand the interaction with Pho80 in the absence of Pho4 suggested that Pho81 might block the ability of the Pho80−Pho85 cyclin−cdk complex to interact with Pho4; on the other hand, the interaction with Pho4 in a Pho80-independent fashion raised the possibility that access to Pho4 might also be blocked by Pho4-bound Pho81; alternatively, Pho4-bound Pho81 might play a positive role as a transcriptional co-activator, mediating interaction between the Pho4 activation domain and the basal transcription machinery. This latter possibility was unlikely, however, since the LexA-Pho81 fusion, in the absence of co-expressed Pho4, failed to activate transcrip-

A

Reporter: *Lex op-CYC-lacZ*

	- PHO4-VP16		+ PHO4-VP16	
	Y700	Y780	Y700	Y780
LexA	<10	<10	<10	<10
LexA-PHO81	120	56	7690	1970
LexA-PHO80	20	20	18410	14280

B

Reporter: *Lex op-CYC-lacZ*

C

Reporter: *Lex op-CYC-lacZ*

Fig. 5. Interaction between Pho81 and Pho4 *in vivo*. (A) Yeast strains Y700 (*ade, leu2, trp1, his3, ura3*) and Y780 (*ade, leu2, trp1, his3, ura3, pho80:HIS3*) were transformed with vectors expressing LexA-Pho81 and Pho4-VP16 fusion proteins and β-galactosidase activity arising from activation of the Lex operator-*CYC-lacZ* reporter was determined. (B) The Pho4 activation domain is inaccessible in the complex with Pho81. Yeast strains Y700 and Y780 were transformed with vectors expressing either Pho4 or the Pho4-VP16 chimera and LexA-Pho81 from the *GAL10* promoter and β-galactosidase activity arising from activation of the Lex operator-*CYC-lacZ* reporter was determined. (C) Pho4 residues N-terminal to amino acid 73 are essential for interaction with Pho81. The WT Pho4-VP16 fusion protein or derivatives containing N-terminal or internal in-frame deletions were expressed from the *GAL10* promoter in yeast strain Y700 together with the LexA-Pho81 chimera and β-galactosidase activity arising from activation of the Lex operator-*CYC-lacZ* reporter determined. The residues at the deletion end-points are indicated. The engineered *Xho*I site is located at amino acid 247 and is situated immediately N-terminal to the Pho4 basic region.

tion to any significant extent; a co-activator fused to LexA might be expected to activate transcription through interaction with the basal transcription machinery. An additional possibility was that Pho4 could interact with Pho81 and the Pho80−Pho85 complex simultaneously with derepression resulting in dissociation of a tripartite Pho81−Pho80−Pho85 complex.

As an initial step towards understanding regulation of Pho4 by Pho81, we asked whether interaction between the LexA-Pho81 fusion protein and a non-VP16-tagged Pho4 protein would result in transcription activation. Any activation obtained would indicate that the Pho4 activation domain remained exposed in the Pho81−Pho4 complex, while no activation would suggest that Pho4 activation domain was inaccessible to the general transcription machinery. To this end, the LexA-Pho81 construct was co-expressed with either the Pho4-VP16 fusion protein or with the WT Pho4 protein. As before, the Pho4-VP16 chimera activated transcription efficiently via the LexA-

Pho81 fusion protein (Figure 5B). Interestingly, no significant activation was observed using WT non-VP16-tagged Pho4, suggesting that the Pho4 activation domain was inaccessible in the complex with Pho81.

To gain a further insight into the potential mode of action of Pho81 we sought to identify the regions of Pho4 required for formation of the Pho4−Pho81 complex. Identification of a specific region of Pho4 required for the interaction with Pho81 would lend support to the idea that the interaction between Pho4 and Pho81 was of biological significance. To this end, a series of mutant Pho4-VP16 proteins was therefore expressed and their ability to interact with the LexA-Pho81 chimera, and thereby activate transcription through the Lex operator, was assessed. The results obtained are shown in Figure 5C. While activation by the WT Pho4-VP16 chimera was extremely efficient, activation by a mutant Pho4 protein (ΔN118) lacking the N-terminal 118 amino acids was severely reduced, >50-fold. Pho4 mutant ΔN200 similarly failed

to activate transcription significantly. These data therefore suggest that residues within the N-terminal 118 amino acids of Pho4 are critical for the interaction with Pho81. Using a series of internal in-frame deletion mutants the requirement for the Pho4 N-terminus was confirmed (Figure 5C). Thus, while the internal deletion mutants Δ118int and Δ74int activated transcription efficiently, and therefore interacted with Pho81, further deletion to amino acid 31 (Δ31int) reduced activation ~4-fold. Mutants ΔN31.Δ200int, lacking both the N-terminal 31 amino acids and internal residues between positions 200 and 247, and ΔN31, lacking only the N-terminal 31 amino acids, failed to activate transcription above the background level obtained by expression of LexA-Pho81 alone. Taken together, the data presented are consistent with sequences in Pho4 both N-and C-terminal to residue 31 but not extending C-terminal to residue 73, being required for the interaction with Pho81. Moreover, since residues essential for the Pho4 activation function lie between amino acids 74 and 99 (Jayaraman *et al.*, 1994), it is clear that Pho81 is not essential for the function of the Pho4 activation domain.

The phosphate switch regulates the interaction of Pho80 and Pho81 with Pho4

The data so far indicate that Pho80 and Pho81 can associate both with Pho4 and with each other. For repression to occur, Pho80 and Pho85 must complex with Pho4, and the evidence available also seemed to indicate that at least a proportion of repressed complexes would also contain Pho81. However, under conditions of phosphate starvation, several possible scenarios could be envisaged, the most likely being that the Pho80−Pho85 complex would dissociate from Pho4. However, since dissociation does not appear to accompany derepression of the Gal4 activation domain by Gal80 (Leuther and Johnston, 1992), there remained a possibility that the Pho80−Pho85 complex remained bound to Pho4 in low phosphate but that the conformation of the complex was altered to permit interaction with Pho2 and to expose the Pho4 activation domain. It was also unclear how Pho81 would behave. If the Pho80−Pho85 complex dissociated, would Pho81 remain bound to Pho4 or would it also be released? To explore the possibility that the interactions between these various components of the PHO regulon were regulated by the phosphate switch, yeast transformed with different combinations of LexA chimeras and VP16 fusion proteins were grown under high or low phosphate conditions and the ability to interact and thereby activate the LexA operator-driven reporter was assayed. The results, which are most readily visualized by performing an *in situ* β-galactosidase assay, are shown in Figure 6. Using this assay it can be seen that under high phosphate conditions Pho4-VP16 can readily associate with LexA-Pho80 and LexA-Pho81, resulting in high levels of β-galactosidase activity derived from the lexA operator-*CYC-lacZ* reporter. No activation was observed in low phosphate, indicating that Pho80 and Pho81 dissociate from Pho4 under de-repressing conditions. In contrast, Pho81-VP16 interacts with LexA-Pho80 irrespective of phosphate concentration, resulting in high β-galactosidase activity on both high and low phosphate plates. Thus, the interaction between Pho81 and Pho80 is insensitive to the phosphate switch while
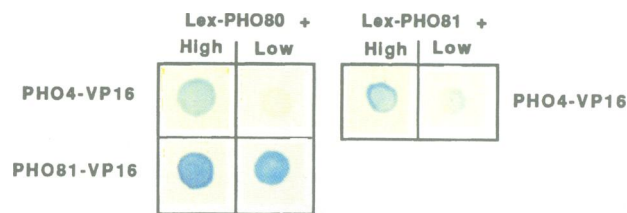
Fig. 6. Interactions between the PHO regulatory proteins in high and low phosphate. The indicated combinations of LexA and VP16 chimeras were transformed into yeast together with the lex operator-*CYC-lacZ* reporter and assayed for their ability to interact, and thereby activate transcription. Yeast grown on high or low phosphate minimal agar plates were assayed using X-gal as a substrate. All the yeast colonies shown were taken from the same high or low phosphate plates and were assayed simultaneously under the same conditions.

that occurring between Pho81 or Pho80 and Pho4 takes place only in high phosphate, when Pho4 is repressed. Consistent with Pho80 dissociating from Pho4 in low phosphate, interaction in the two-hybrid assay between Pho85 and Pho4, which is dependent on Pho80, is only observed in high phosphate (data not shown).

Discussion

The integration of growth and division with the nutritional status of the cell must involve the communication between multiple different regulatory pathways designed to elicit the coordinated control of transcription in response to a variety of nutritional signals. In this respect, the regulation of the yeast acid phosphatase gene, *PHO5*, provided us with an opportunity to understand how the response to an essential nutrient is mediated by complex interactions between several regulatory proteins in a genetically well characterized system. The results obtained provide a significant insight into transcription regulation by an essential nutrient, together with the mechanism underlying derepression mediated by the cyclin-associated protein Pho81.

Transcription regulation by the phosphate switch

The inability of the Pho4-VP16 chimera to activate the *PHO5* UAS in high phosphate provided a strong indication that the ability of Pho4 to bind DNA is regulated by the phosphate switch. This conclusion is supported by evidence obtained by *in vivo* footprinting, which indicates that under repressing, high phosphate conditions, Pho4 is not bound to the *PHO5* UAS (Venter *et al.*, 1994). The inability of Pho4 to bind in high phosphate reflects the regulation of its interaction with Pho2. The homeobox protein Pho2 is required for Pho4-dependent expression of the acid phosphatase gene *PHO5*, and we have shown that Pho4 interacts with Pho2 in a two-hybrid assay and that the two proteins act cooperatively on the *PHO5* UAS, with the interaction between them being regulated by the phosphate switch. Interestingly, the region of Pho4 between amino acids 190 and 230 which we have determined to be sufficient for interaction with Pho2 maps to the 'oligomerization' domain described by Ogawa and Oshima (1990), which was identified as a region of Pho4 which could act *in trans* to inhibit the ability of the endogenous Pho4 protein to activate transcription. It was concluded that expression of this domain probably

inhibited the ability of Pho4 to oligomerize. However, given our results, it seems clear that rather than mediating oligomerization of Pho4, the *trans*-inhibition would be explained by its capacity to interact with Pho2 and deplete the pool of Pho2 free to bind the *PHO5* UAS cooperatively with endogenous Pho4.

While the ability of Pho4 to interact with Pho2 is an important element in regulating its activity on the *PHO5* UAS, several lines of evidence suggest that the Pho80 and Pho85 proteins can repress Pho4 by two different mechanisms. Thus, Pho80 can repress DNA-bound Pho4, even in the absence of Pho85 (Madden *et al.*, 1990), indicating that Pho80 itself can repress by a Pho85-independent mechanism, an idea supported by the fact that the constitutive activation of the *PHO5* promoter in a *pho85* disruption strain is only 15% of that obtained in a *pho80* disruption strain (Lemire *et al.*, 1985; our unpublished observations). One conclusion to be drawn from these observations is that while Pho80 is absolutely required for repression, Pho85 is only required for full repression.

A further clue to the role of the Pho80–Pho85 repressor comes from an examination of the Pho4-dependent promoter of the alkaline phosphatase gene, *PHO8*. While Pho2 is required for the expression of *PHO5*, it is not required for activation of the *PHO8* promoter (Kaneko *et al.*, 1985). Our results which indicate that DNA binding by Pho4 is regulated by its interaction with Pho2, are therefore entirely compatible with the observation that Pho4 remains associated with the *PHO8* promoter in high phosphate as assayed using *in vivo* footprinting techniques (W.Hörz, personal communication). If Pho4 remains bound to the *PHO8* promoter in high phosphate, it is evident that, in addition to the regulation of the interaction with Pho2 on the *PHO5* UAS, repression must also involve the inactivation of the Pho4 activation domain. This conclusion is supported by the fact that a LexA-Pho4 chimera is efficiently repressed by Pho80 (Jayaraman *et al.*, 1994). Thus, the ability of Pho80 to repress directly appears to reflect its capacity to mask the Pho4 activation domain rather than any inhibition of DNA binding through regulation of the interaction with Pho2.

Taken together, this information is consistent with a model (see Figure 7 and below) in which the interaction between Pho4 and Pho2 is required for Pho4 to bind the low affinity P1 site in the nucleosome-free gap on the *PHO5* UAS, but not high affinity site in the *PHO8* UAS. Interaction between Pho4 and Pho2 is inhibited by phosphorylation of Pho4, presumably, but not necessarily, by the Pho80–Pho85 cyclin–cdk complex which can also mask the Pho4 activation domain. This model would account for the differential behaviour of Pho4 on the *PHO5* and *PHO8* promoters, the dual role of the Pho80–Pho85 complex and the requirement for Pho2 on the *PHO5* UAS. We are currently assessing the viability of this model by examining more closely the Pho4–Pho2 interaction *in vivo* using *pho80* and *pho85* mutants.

The capacity for the interaction between Pho2 and Pho4 to be regulated by the phosphate switch is potentially extremely significant. Pho2 is involved in regulation of a variety of genes, including *ADE1*, *TRP4*, *HIS4* and the *HO* gene (Arndt *et al.*, 1987; Braus *et al.*, 1989; Tice-Baldwin *et al.*, 1989; Daignan and Fink, 1992; Brazas
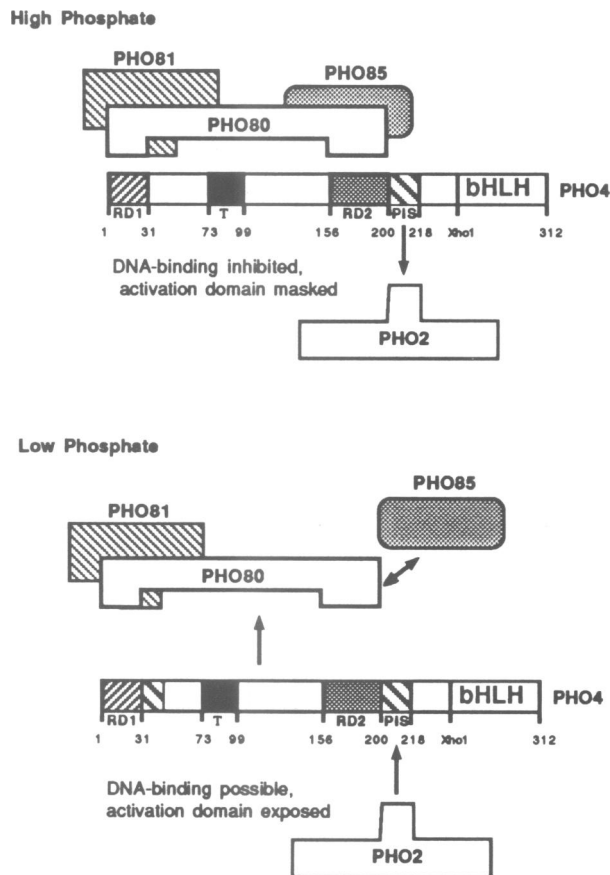
Fig. 7. A model describing the interactions between Pho4 and Pho2, Pho80 and Pho81 under high and low phosphate conditions. Under high phosphate conditions Pho4 is repressed by the Pho80–Pho85 cyclin–cdk complex, which masks the Pho4 activation domain through Pho80 interaction with the Pho4 repression domains, RD1 and RD2. In high phosphate, the interaction between Pho4 and Pho2 through the Pho2 interacting sequence (PIS) is inhibited, preventing Pho4 binding to the low affinity P1 element in the *PHO5* UAS. A proportion of the pool of Pho4–80–85 complexes also contains Pho81, which can interact both with Pho4 and with Pho80. The complexes containing Pho81 will be primed to respond to phosphate starvation. In low phosphate conditions, Pho81 mediates the dissociation of the Pho80–Pho85 complex from Pho4, enabling Pho4 to interact with Pho2, bind DNA and activate transcription. It is also possible that Pho85 may exchange between Pho80 and its related cyclins.

and Stillman, 1993a,b) where it acts cooperatively with other DNA binding transcription factors such as Bas1 (Arndt *et al.*, 1987; Tice-Baldwin *et al.*, 1989; Daignan and Fink, 1992) and Swi5 (Brazas and Stillman, 1993a,b). Since both Pho2 and Pho85 appear to be involved in several cell functions (Gilliquet and Berben, 1993) it is interesting to speculate that the interaction between Pho2 and other DNA binding partners may also be regulated by cyclin–cdk complexes, perhaps involving Pho85 and the Pho80-related cyclins Hcs26 and OrfD or by other nutritional signals, thereby allowing the integration of several aspects of cell physiology with the cell cycle. We are currently investigating the possibility that Pho2 plays a pivotal role in coordinating the transcriptional response to multiple physiological signals.

The role of Pho81 in mediating the phosphate switch

In addition to its capacity to bind DNA cooperatively with Pho2, transcription activation by Pho4 requires two

addtional functions – the ability to activate transcription through the basal transcription machinery, and the ability to be derepressed. While repression of Pho4 is mediated primarily by the Pho80–Pho85 cyclin–cdk complex, derepression/activation requires the Pho81 regulator (Toh-e *et al.*, 1973; Creasy *et al.*, 1993). Several lines of evidence suggest that Pho81 is required for derepression rather than true activation. Thus, Pho81 does not interact with the Pho4 activation domain and therefore does not appear to play a role as a transcriptional coactivator. Pho81 can interact both with Pho4 and with Pho80. The interaction of both Pho80 and Pho81 with Pho4 is sensitive to the phosphate switch, interaction with Pho4 only occurring in high phosphate repressing conditions. In contrast, interaction between Pho81 and Pho80 appears to be independent of phosphate levels, suggesting that Pho80 is the primary target for regulation by Pho81. Consistent with this, overexpression of Pho80 can partially counteract the effects of Pho81 constitutive mutants (Creasy *et al.*, 1993). Moreover, *PHO81* is the only regulatory gene in the PHO regulon which is sensitive to phosphate levels, with both Pho4 and Pho2 being required for its transcription (Yoshida *et al.*, 1989). Although we cannot exclude the possibility that Pho80 and Pho81 do not bind Pho4 simultaneously, the available evidence is consistent with a model (Figure 7) in which, in high phosphate, Pho4 is present in a complex containing Pho80–Pho85. A proportion of these complexes will also contain Pho81. When conditions of phosphate starvation are encountered, intracellular phosphate levels will fall and the Pho81 protein, acting as a phosphate sensor, will mediate the release of the Pho80–Pho85 complex from Pho4. Pho4 would then be free to interact with Pho2 and activate the *PHO5* promoter as well as increase transcription from the *PHO81* gene. This would then result in a further increase in Pho81 protein and consequently replenish the pool of Pho81-primed Pho4–80–85 complexes responsive to intracellular phosphate levels. Whether Pho81 is directly sensitive to phosphate, or whether the phosphate switch is mediated in the first instance by phosphorylation of Pho81 in response to intracellular phosphate levels remains unclear.

Pho81 can be viewed both as an anti-repressor of Pho4 function and as an inhibitor of the Pho80–Pho85 cyclin–cdk complex. By binding to the Pho80 cyclin Pho81 clearly differs from the p21 family of cdk inhibitors (Gu *et al.*, 1993; Gyuris *et al.*, 1993; Harper *et al.*, 1993; Serrano *et al.*, 1993; Xiong *et al.*, 1993; Polyak *et al.*, 1994) which interact with the cdk component of the cyclin–cdk complexes. Whether Pho81 in any way resembles Far1, which inhibits START function by binding and inactivating the complexes formed between the G_1 cyclins Cln1, Cln2 and Cln3 with Cdc28 (Peter *et al.* 1993; Tyers and Futcher, 1993; McKinney *et al.*, 1993), remains to be seen, since it is not yet clear which component of the Cln–Cdc28 complex is recognized by Far1 and it remains to be established whether Pho81 can also regulate the action of a variety of cyclin–cdk complexes. However, a wider role for Pho81 in controlling the cellular response to phosphate levels appears an attractive possibility; some mechanism must exist for the exchange of information between regulatory pathways in order to coordinate the cellular response to different

nutritional signals. One possibility that we find particularly attractive is if members of a family of Pho81-like proteins were each able to respond to different nutritional signals by regulating a family of Pho80-like cyclins. These cyclins, possibly in association with Pho85, would in turn target the Pho2-interacting factors controlling the transcription of genes required for the synthesis or maintenance of essential nutrients for the cell. While we accept that this represents a considerable speculation, it nevertheless provides a framework for future studies designed to dissect the molecular mechanisms underlying the transcriptional response to nutritional signals.

Finally, while the models for repression and activation presented here are supported by the data available, additional work will be necessary to assess their validity. Precisely how the response to phosphate is coupled to other nutritional signals, and which mechanism operates at which stage in the cell cycle will require further investigation.

Materials and methods

Yeast strains and media

The *Saccharomyces cerevisiae* strains Y704, Y780, Y785 and Y781 used were isogenic with strain Y700 (a, *ade2-1, trp1-1, can1-100, leu2-3, leu2-112, his 3-11,15, ura3*) but contained *HIS3* disruptions of the *PHO4, PHO80, PHO85* and *PHO81* genes respectively. The Y700, Y704 and Y780 strains have been described previously (Fisher *et al.*, 1991; Jayaraman *et al.*, 1994). Strain Y785 was made by insertion of the *HIS3* gene as a *Bam*HI fragment (a clone containing the *HIS3* gene as a *Bam*HI fragment was kindly provided by Jane Mellor) into the unique *Bgl*II site of the cloned *PHO85* coding sequence and subsequent disruption of the *PHO85* gene in Y700 by homologous recombination *in vivo*. A similar approach was used to construct the Y781 strain, with insertion of the *HIS3* gene as a *Bam*HI fragment between the two *Bgl*II sites in the *PHO81* coding sequence.

Yeast cultures were grown at 30°C in either YPD (1% yeast extract, 2% glucose, 1% peptone) or in minimal medium (0.67% yeast nitrogen base, 1% glucose) supplemented with the appropriate amino acids (0.002%) as required. For the experiments involving high or low phosphate minimal essential medium was used but with phosphate levels adjusted to 7.4 mM (high) or 3.7 μM (low).

Yeast transformations and β-galactosidase assays

Yeast transformations were performed following the procedure of Hinnen *et al.* (1978). For liquid β-galactosidase assays, yeast colonies were picked into 6 ml glucose minimal medium supplemented with the appropriate amino acids and grown for 24–48 h. The cells were then pelleted by low speed centrifugation, resuspended in 1 ml minimal medium and 100 μl transferred into 6 ml fresh galactose or glucose minimal medium as indicated. After 18 h at 30°C the cells were harvested by centrifugation, washed in H_2O and resuspended in 100 μl buffer containing 0.1 M Tris–HCl pH 7.5 and 0.05% Triton X-100 before freezing. Assays for β-galactosidase activity were then performed as described (Harshman *et al.*, 1988). All experiments were repeated several times.

For the *in situ* assays, yeast were washed in low phosphate medium, resuspended at an equivalent density and spotted onto nitrocellulose filters placed on high or low phosphate glucose or galactose minimal agar plates. The plates were then placed at 30°C overnight. The filters were subsequently removed, snap frozen on an aluminium foil float in a liquid nitrogen bath and placed on Whatman 3MM paper saturated with Z-buffer (Harshman *et al.*, 1988) containing X-gal. The colour was allowed to develop at 30°C.

Acid phosphatase assays

For liquid assays, rAPase activity was measured using whole cells grown in high phosphate medium as a source of enzyme and *p*-nitrophenol phosphate (Sigma 104) as substrate as described (Toh-e *et al.*, 1973). Essentially yeast were grown overnight in 6 ml high phosphate glucose minimal medium (7.4 mM phosphate), centrifuged and washed in low phosphate (3.7 μM) minimal medium and then resuspended in 1 ml low

phosphate medium. An aliquot was then transferred to a fresh 20 ml high or low phosphate medium and grown to an optical density at 600 nm of between 0.3 and 0.6, washed in acetate buffer (60 mM, pH 4.3), resuspended in 100 μl acetate buffer and 30 μl added to a reaction mix comprising 0.5 ml acetate buffer containing 0.64 mg/ml p-nitrophenol phosphate. The reaction was then incubated at 30°C for an appropriate time before being stopped by the addition of an equal volume of a saturated Na_2CO_3 solution. Cells were removed by centrifugation before measuring the OD_{420}. The relative rAPase activity was determined by the formula $A_{420}/A_{600} \times t$, where t is the time in min.

For the *in situ* rAPase assay, yeast were plated at equal density on high or low phosphate glucose minimal agar containing appropriate amino acids and grown overnight at 30°C. To assay rAPase activity, 4 ml of 1% agar in 60 mM acetate buffer (pH 4.3) containing 0.5 mg/ml α-naphthyl acid phosphate and 5 mg/ml fast garnet GBC salt was used as an overlay and the yeast incubated at 20°C for 12 min.. Note that while the yeast were seeded at an equivalent concentration, growth on high phosphate agar is considerably greater, accounting for the increased size and density of the colonies assayed compared with those on low phosphate plates. Quantitation by liquid phosphatase assay in parallel using whole cells as a source of enzyme and p-nitrophenol phosphate as substrate showed that the combination of VP16-Pho2 and Pho4ΔN156 induced rAPase activity at least 15-fold greater than the expression of VP16-Pho2 alone in low phosphate.

Expression vectors and reporters

The *PHO5* UAS *CYC-lacZ* reporter has been described previously (Fisher *et al.*, 1991), as has the reporter containing the *LexA* operator (Jayaraman *et al.*, 1994). The vectors expressing Pho4 WT, the Pho4-VP16 fusion proteins and the N-terminal and internal in-frame deletion mutants from the *GAL10* promoter have also been described before (Jayaraman *et al.*, 1994). Other expression vectors using the *GAL10* promoter all contain a unique *Bgl*II or *Sal*I cloning site located downstream from the *GAL10* promoter and upstream from the *PGK* terminator. The *GALp-PGKt* cassette was inserted as an *Eco*RI–*Sal*I fragment into either high or low copy number pRS vectors containing the appropriate selectable markers (Sikorski and Hieter, 1989). The *PHO4* promoter vector contains the *PHO4* promoter, as a 372 bp 5′ *Eco*RI–3′ *Bgl*II fragment, placed upstream from the *PGK* transcription terminator sequence (*Bgl*II–*Sal*I fragment), with the entire *PHO4-PGK* sequence inserted between the RI and *Sal*I sites of pRS315 (Sikorski and Hieter, 1989). In the PHO4-VP16 constructs, Pho4 amino acids 1–310 are fused in-frame to the C-terminal 80 amino acids of the herpes simplex virus VP16 transactivator. The PHO4-VP16 coding sequences were inserted as a *Bam*HI fragment into the unique *Bgl*II site downstream from the *PHO4* promoter. To construct pVP16-PHO2, the *PHO2* coding sequences were inserted in-frame into a *Bgl*II site at the 3′end of a *Bam*HI fragment encoding the 80 amino acid VP16 activation domain modified to contain a 5′ ATG and lack the natural VP16 stop codon. The *VP16-PHO2* fusion was then inserted as a *Bam*HI fragment into the *Bgl*II cloning site either of a vector containing the *PHO4* promoter and PGK terminator inserted into pRS314, or downstream from the Gal promoter in pRS313. The *PHO81* coding sequences were isolated by PCR from yeast genomic DNA so that the primers used placed unique *Sal*I restriction sites at the 5′ and 3′ termini. To make VP16-PHO81, the *PHO81* coding sequences as a *Sal*I fragment were inserted into an *Xho*I site of a pUC.VP16 clone which contained the same VP16 sequence as was fused to the *PHO2* coding sequence with the exception that the *PHO81-VP16* fusion could be excised as a *Sal*I fragment. The *PHO81-VP16* coding sequences were then inserted into a unique *Sal*I site downstream from the *GAL10* promoter in pKV701 (Fisher *et al.*, 1991), containing a *Sal*I linker inserted into the unique *Bgl*II cloning site.

The LexA expression vector, V44ER.Lex (*TRP⁺*, CEN/ARS), is derived from pV44ER (R.Treisman) by deletion of the VP16 activation domain, and contains the *GAL* UAS upstream of the basal *CYC* promoter fused to the coding sequences for the bacterial LexA repressor. A polylinker containing unique *Xho*I, *Eco*RI, *Nco*I and *Cla*I is located between the LexA coding sequences and the *CYC* terminator. The *PHO80* coding sequences were inserted as an *Xho*I PCR product in-frame and C-terminal to LexA and upstream of the *CYC* terminator sequence as described previously (Jayaraman *et al.*, 1994). Similarly, the LexA-Pho81 expression vector was made by insertion of the *PHO81* coding sequences as a *Sal*I fragment into the unique *Xho*I site of V44ER.Lex. An additional LexA expression vector, V44ER.Lex.BglII, containing a unique *Bgl*II site adjacent and 3′ to the *Xho*I site was also constructed by ligation of a double-stranded oligonucleotide containing a *Bgl*II site between the *Xho*I and *Eco*RI sites of V44ER.Lex. In this

vector the *Xho*I and *Eco*RI sites are retained. The *PHO85* coding sequences were inserted in-frame with the LexA coding sequence into the *Bgl*II site of V44ER.Lex.BglII site as a *Bam*HI fragment. The *PHO2* coding sequences as a *Bam*HI fragment were also cloned in-frame with and C-terminal from the LexA coding sequences into the unique *Bgl*II site of pV44ERLex.BglII.

In the epitope-tagged expression vectors, three different epitopes were used as N-terminal tags: the SV5 epitope includes amino acids 95–108 of the P and V proteins from the SV5 virus (Hanke *et al.*, 1992) and comprises the sequence M-GKPIPNPLLGLDST; the Myc epitope 9E10 is derived from residues within the leucine-zipper of the c-myc protein (Evan *et al.*, 1985; Munro and Pelham, 1987) and comprises the sequence M-EQKLISEEDLNM; and the HA epitope is derived from the influenza virus haemagglutinin protein (Wilson *et al.*, 1984) and comprises the sequence M-YPYDVPDYAG. The coding sequence for each epitope was inserted downstream from the *GAL10* promoter by oligonucleotide-directed PCR with the 5′ PCR primer (agaactagtTGTGGAAATGTAAA-GAGCCCC) placing an *Spe*I site upstream of the *GAL* promoter and the 3′ PCR primers encoding the relevant epitopes and GAL promoter sequences together with 3′ *Xho*I and *Bgl*II sites to facilitate subsequent cloning. Each PCR product was inserted as *Spe*I–*Bgl*II fragments upstream of the *PGK* terminator in pRS315 or pRS314. The *PHO80* coding sequences were then inserted downstream from the *GAL* promoter as a *Bam*HI fragment into the unique *Bgl*II site of pRS315.KV.SV5 while the *PHO81* coding sequences were inserted downstream from the *GAL* promoter in pRS314.KV.Myc.

Co-immunoprecipitations and Western blotting

For the co-immunoprecipitation experiments we used a protocol modified from that of Kaffman *et al.* (1994). Essentially 200 ml yeast culture grown to an OD_{600} of 0.6 were harvested and washed with 15 ml PBS. All subsequent steps were performed at 4°C. The yeast cells were resuspended in 350 μl HSB buffer [45 mM HEPES–KOH pH 7.5, 400 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5% NP-40, 2 mM DTT, 2 mM benzamide, 1 mM PMSF, leupeptin (1 μg/ml), pepstatin (1 μg/ml), 10 mM NaF, 10 mM calyculin A] and 300 μl equivalent acid washed glass beads added. The cells were lysed on a vibramax for 5 min and spun in a microfuge for 5 min. The supernatant was transferred to a new tube and centrifuged for a further 15 min. This supernatant was used as the lysate.

150 μl lysate were rolled with 10 μl protein A–Sepharose beads (precleared for 1 h with normal rabbit serum) together with either anti-SV5 (Hanke *et al.*, 1992) or anti-Myc (9E10) epitope antibody (Evan *et al.*, 1985) and 0.5 μl normal rabbit serum. After incubation for 4 h the beads were then washed four times with 200 μl PBS containing 150 mM NaCl, 10 mM Na phosphate pH 7.4 and 1% NP-40 and once with PBS. Finally 25 μl 'boiling blue' (50 mM Tris–HCl pH 6.8, 600 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) were added, the samples boiled for 2 min and 10 μl loaded onto a mini 14% SDS–PAGE gel. After transfer to nitrocellulose, the blot was probed sequentially with the indicated antibodies in 10% calf serum/PBS. Alkaline phosphatase coupled to anti-rabbit, anti-rat or anti-mouse immunoglobulin G, as appropriate, was used as the second antibody.

Acknowledgements

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