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# The activation domain of a basic helix-loop-helix protein is masked by repressor interaction with domains distinct from that required for transcription regulation

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**While there are many examples of protein-protein interactions modulating the DNA-binding activity of transcription factors, little is known of the molecular mechanisms underlying the regulation of the transcription activation function. Using a two-hybrid system we show here that transcription repression of the basic domain/helix-loop-helix factor PHO4 is mediated by complex formation with the PHO80 repressor. In contrast to other systems, such as inhibition of GAL4 by GAL80 or of p53 by MDM2, where repression is mediated by direct interaction at regions overlapping the transcription activation domain, interaction with PHO80 involves two regions of PHO4 distinct from those involved in transcription activation or DNA-binding and dimerization. The possibility that repression of PHO4 by PHO80 may represent a general mechanism of transcription control, including regulation of the cell-type-specific transcription activation domain of c-Jun, is discussed.**

*Key words:* helix-loop-helix/PHO4/PHO80/protein-protein interaction/transcription repression

## Introduction

The isolation of a multitude of genes encoding eukaryotic transcription factors has revealed that many may be grouped into families sharing homology across domains required for DNA-binding and dimerization and that members of a given family may exhibit similar or identical binding specificity (Mitchell and Tjian, 1989; Harrison, 1991). Given that multiple factors, each able to bind the same sequence, may be present in the same cell, mechanisms must exist to maintain the regulatory specificity required for the precise and co-ordinated regulation of gene expression essential for differentiation, cell growth and division, and the rapid response of genes to developmental and environmental stimuli. While a variety of mechanisms may operate to modulate the DNA binding or function of transcription factors (Jones, 1990; Karin, 1990, 1991), it is evident that differential protein-protein interactions play a major role in determining regulatory specificity. However, although there are many examples of protein-protein interactions promoting or preventing DNA-binding by transcription factors, much less is known of mechanisms underlying the regulation of the transcription activation function. This lack of information arises both because of the complexity of the transcription activation process and from the difficulties involved in the reconstitution of transcription regulation using purified components necessitated by the limitations of using a genetic approach with mammalian cells.

In contrast to mammalian systems, the genetics of *Saccharomyces cerevisiae* has facilitated the identification of a range of genes encoding positive and negative regulatory proteins many of which share structural features with mammalian transcription factors. For example, *S.cerevisiae* provides an excellent system for understanding the molecular mechanisms underlying regulated gene expression by the basic domain/helix-loop-helix (bHLH) family of transcription factors; at least four bHLH proteins, PHO4 (Ogawa and Oshima, 1990), CPF1 (Baker and Masison, 1990; Cai and Davis, 1990; Mellor *et al.*, 1990); INO2 (Nikoloff *et al.*, 1992) and INO4 (Hoshizaki *et al.*, 1990), have been identified, each able to bind the same core CANNTG motif but acting to regulate distinct sets of genes. Thus, the situation in yeast clearly parallels that in mammalian cells were multiple bHLH proteins having the potential to recognize the same or similar sequences are found in the same cell at the same time. While some bHLH proteins may possess subtly different DNA-binding properties (Fisher and Goding, 1992), other mechanisms must operate to regulate differentially their transcription activation potential. Regulatory mechanisms operating in yeast are likely to be conserved in evolution and may serve as paradigms for those functioning in mammalian cells.

Activation of the yeast acid phosphatase gene *PHO5* by the bHLH transcription factor PHO4 (Vogel *et al.*, 1989; Ogawa and Oshima, 1990) represents an excellent system for examining transcription regulation: activation by PHO4, which can bind DNA as a homodimer and which is constitutively present in the cell (Koren *et al.*, 1986; Legrain *et al.*, 1986; Yoshida *et al.*, 1989b), is prevented under high phosphate conditions by the products of the *PHO80* (Madden *et al.*, 1990) and *PHO85* genes (Uesono *et al.*, 1987); under low phosphate conditions PHO4 is de-repressed and is able to activate transcription (Lemire *et al.*, 1985; Yoshida *et al.*, 1989a). Although genetic evidence suggests that PHO80 interacts directly with PHO4 (Okada and Toh-e, 1992), the molecular mechanisms underlying repression remain unknown. Thus, the evidence to date does not distinguish between PHO80 acting to inhibit the PHO4 transcription activation function or its ability to bind DNA. Neither is it clear whether repression is mediated by post-translational modification of PHO4 by PHO80 or by complex formation between the two proteins. Understanding the molecular mechanisms underlying repression of PHO4 by PHO80 should provide a fundamental insight into how the activity of a sequence-specific transcription factor may be controlled.

In this report we show, using a two-hybrid system, that



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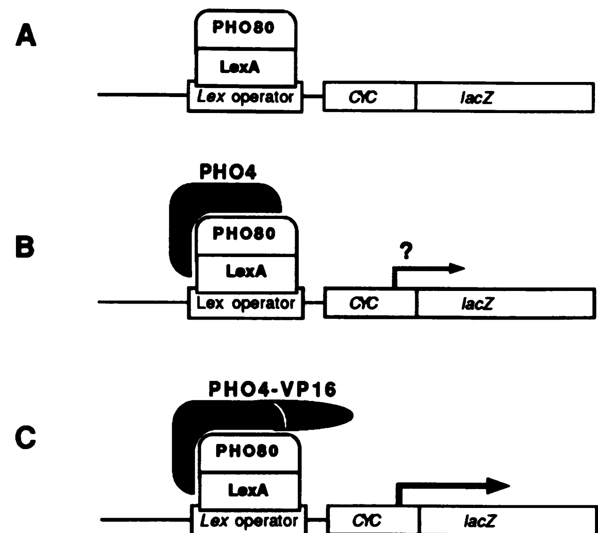
the ability of PHO4 to activate transcription is prevented by an association with PHO80 *in vivo*. Unlike repression of other bHLH proteins which is mediated by inhibition of DNA-binding, repression by PHO80 does not involve the PHO4 bHLH domain. In contrast, the evidence suggests a mechanism involving masking of the PHO4 activation domain by PHO80. Unlike repression of GAL4 by GAL80 (Ma and Ptashne, 1987; Salmeron *et al.*, 1990; Leuther *et al.*, 1993) or p53 by MDM2 (Oliner *et al.*, 1993) where the requirements for transcription activation and repression overlap, the PHO4 transcription activation region does not participate in interaction with the PHO80 repressor. Rather, interaction can be mediated independently by regions of PHO4 both N- and C-terminal to the activation domain. Inhibition of PHO4 by PHO80 may be taken as an example for an alternative mode of regulation of the transcription activation potential of a sequence-specific transcription factor.

## Results

### *PHO4 and PHO80 interact in vivo*

Repression of PHO4 by PHO80 must involve either inhibition of the ability of PHO4 to bind DNA or its ability to interact with other components of the transcription machinery and could be mediated either by formation of a protein–protein complex or by post-translational modification of PHO4 induced by PHO80. Sequence analysis of PHO80 failed to provide clues to any enzymatic function (Madden *et al.*, 1990). We therefore devised a method based on the two-hybrid system (Fields and Song, 1989; Chien *et al.*, 1991) to examine the possibility that PHO80 function was mediated by direct complex formation with PHO4. The system used is depicted in Figure 1. Briefly, the PHO80 coding sequences were fused in-frame to those encoding the bacterial LexA repressor. Expression of this hybrid protein from the inducible *GAL10* promoter should not activate transcription from a *CYC–lacZ* reporter under the control of the *lexA* operator since it contains no activation domain (Figure 1A). If the PHO4 protein were co-expressed with the LexA–PHO80 chimera, two results would be possible: if the PHO4 protein could interact with PHO80 *in vivo* but interaction left the PHO4 transcription activation domain exposed, then transcription from the *lexA* operator–*lacZ* reporter would occur; in contrast, no transcription would occur either if PHO4 did not complex with PHO80, or if interaction did occur but the PHO4 activation domain was masked (Figure 1B). To distinguish between these possibilities, we also fused to the C-terminus of PHO4 a second activation domain, the C-terminal 80 amino acids from the herpes simplex virus VP16 (Vmw65) protein, which is transcriptionally active in yeast (Cousens *et al.*, 1989). This chimeric PHO4 protein is functional and can readily activate expression from the natural PHO4 targets in the *PHO5* upstream activation sequence (UAS; see below). We reasoned that, even if interaction with PHO80 masked the PHO4 activation domain, the presence of the additional activation domain would most likely escape regulation by PHO80 and allow activation of the reporter (Figure 1C). The results obtained from using this system are shown in Figure 1D.

Initial experiments verified that neither the PHO4–VP16 chimera nor PHO4 could activate transcription from the *lex* operator *CYC–lacZ* reporter. Similarly, no activation




D

Reporter: *Lex op-CYC-lacZ*

| | + PHO4-VP16 | + PHO4 | -PHO4 |
|---|---|---|---|
| – | <10 | <10 | <10 |
| LexA | <10 | <10 | <10 |
| LexA-PHO80 | 18420 | 70 | <10 |

**Fig. 1.** PHO4 and PHO80 interact *in vivo*. (**A**) Expression of LexA–PHO80 chimera will bind to the *lexA* operator but will not activate transcription from the *CYC–lacZ* reporter. (**B**) Co-expression of PHO4 with the LexA–PHO80 chimera will fail to activate transcription from the reporter either if PHO4 and PHO80 do not interact or if interaction masks the PHO4 activation domain. Activation will occur if PHO4 and the LexA–PHO80 chimera interact and the PHO4 activation domain remains exposed. (**C**) Co-expression of LexA–PHO80 together with PHO4–VP16 chimera comprising the VP16 activation domain fused in-frame to the C-terminus of PHO4, will activate transcription if PHO4 and PHO80 interact, irrespective of whether or not the PHO4 activation domain is exposed. (**D**) β-Galactosidase levels obtained from the *lexA* operator *CYC–lacZ* reporter by expressing the combinations of proteins indicated. The PHO4 proteins were expressed from the high copy number pKV701 vector and the LexA proteins from the high copy number pRS vector. The pKV701 vector was used for the –PHO4 controls.

was observed by expressing either the LexA repressor or the LexA–PHO80 fusion protein alone. In contrast, co-expression of PHO4–VP16 with the LexA–PHO80 chimera resulted in highly efficient transcription activation, strongly suggesting that the two proteins interacted *in vivo*. Interaction was dependent on PHO80 since no activation was observed if the PHO4–VP16 fusion protein was co-expressed with LexA.

Although it was evident that PHO4 and PHO80 could interact, it was unclear what this implied for the function of PHO4; interaction with PHO80 could either inhibit PHO4 DNA-binding or mask its activation domain. If interaction with PHO80 left the PHO4 activation domain exposed then co-expression of PHO4 with the LexA–PHO80 chimera should result in efficient activation from the reporter. On the other hand, an inability to activate would suggest that the PHO4 activation domain was masked in the PHO4–

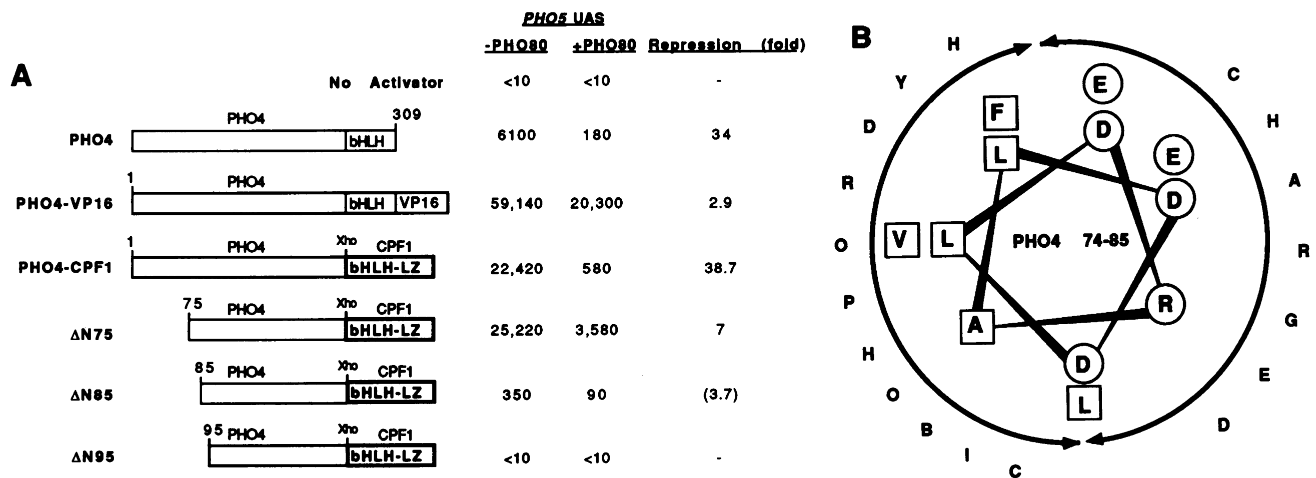



**Fig. 2.** (**A**) The PHO4 DNA-binding domain is not required for repression by PHO80. PHO4, PHO4–VP16 or PHO4–CPF1 fusions were expressed either alone or together with PHO80 and β-galactosidase activity measured from a *PHO5* UAS *CYC–lacZ* reporter. The levels of β-galactosidase activity shown are given to the nearest 10 units. (**B**) Helical wheel analysis of PHO4 amino acids 74–85 encoding a putative amphipathic α-helix. The incremental angle is 100°. Hydrophobic residues are boxed and charged residues circled.

PHO80 complex. To distinguish between these possibilities we co-expressed the LexA–PHO80 fusion protein with PHO4. In contrast to the highly efficient transcription obtained using the PHO4–VP16 chimera, expression of PHO4 and LexA–PHO80 resulted in an extremely low level of activation, at least 90-fold lower than that obtained with PHO4 activating transcription from the *PHO5* UAS (see Figure 2A). Taken together, these results strongly suggest that PHO4 and PHO80 interact *in vivo* and that the PHO4 activation domain is masked as a consequence.

### *Repression by PHO80 requires sequences outside the bHLH*

The data presented so far demonstrate that PHO4–VP16 and LexA–PHO80 interact *in vivo* and that the interaction is sufficiently stable to promote transcription. Since the interaction between PHO4 and the LexA–PHO80 fusion activated transcription very weakly, it was likely that the PHO4 activation domain was masked. However, before any definitive conclusions could be drawn as to possible mechanisms for repression by PHO80 it was necessary both to rule out an effect of PHO80 on the bHLH domain of PHO4 required for DNA-binding and dimerization and to identify precisely the region of PHO4 required for transcription activation. To do this we expressed a series of wild-type (WT) and mutant PHO4 proteins and asked whether they could activate transcription from the *PHO5* UAS placed upstream from the *CYC–lacZ* reporter and whether they were repressible by co-expression of a WT PHO80 protein. The results are shown in Figure 2A.

Expression of PHO4 efficiently activated the *PHO5* UAS, at least 600-fold relative to the levels obtained in the absence of PHO4. Co-expression of PHO80 repressed transcription by ~34-fold. In contrast, the PHO4–VP16 fusion protein activated transcription almost 10-fold more efficiently than PHO4 but was repressed a maximum of 3-fold. Since the PHO4–VP16 fusion efficiently interacts with PHO80 (see Figure 1) but retains its ability to activate transcription from the *PHO5* UAS, it is unlikely that direct repression by PHO80 is mediated by an inhibition of PHO4 DNA-binding. The low level of repression observed presumably reflects the fact that activation by the PHO4–VP16 fusion comprises a contribution from both the PHO4 and VP16 activation domains and is most easily explained if interaction with PHO80 masked the PHO4, but not the VP16 activation domain.

To provide further evidence against the bHLH domain of PHO4 being a target for PHO80 we also replaced the PHO4 bHLH domain with a heterologous bHLH–leucine zipper from CPF-1, which can also recognize the CACGTG motif bound by PHO4 (Fisher and Goding, 1992). The PHO4–CFP1 chimera activated transcription from the *PHO5* UAS some 3- to 4-fold better than PHO4 but was nevertheless repressed at least as efficiently, almost 40-fold, by co-expression with PHO80 (Figure 2A). Taken together with the inability of PHO80 to repress PHO4–VP16-mediated activation of the *PHO5* UAS, these results indicate that PHO80 neither interacts with the PHO4 DNA-binding domain nor inhibits the ability of PHO4 to bind DNA.

### *A potential acidic amphipathic helix is required for efficient transcription activation by PHO4*

The available evidence suggests that PHO80 represses transcription by masking the PHO4 activation domain rather than acting to inhibit the ability of PHO4 to bind DNA. Although previous reports have demonstrated that the PHO4 activation domain lies N-terminal to amino acid 109 (Ogawa and Oshima, 1990) or 118 (Fisher *et al.*, 1991), it was necessary to define the requirements for transcription activation more precisely. Examination of the primary amino acid sequence within the N-terminal 109 amino acids of PHO4 revealed a highly acidic region between amino acids 74 and 85 predicted to form an acidic amphipathic α-helix (Figure 2B). Whether any activation domain in fact adopts an α-helical conformation has yet to be resolved (O'Hare and Williams, 1992; Leuther *et al.*, 1993; Van Hoy *et al.*, 1993). However, since similar predictions have been made for other activation domains, including that of VP16 (Cousens *et al.*, 1989), we asked whether this region was responsible for the PHO4 transcription activation function. To this end, we introduced a small series of N-terminal deletions into the PHO4–CPF1 fusion protein and assayed

their ability to activate the *PHO5* UAS or be repressed by PHO80. The PHO4–CPF1 chimera was used as a background since it activated transcription better than the WT PHO4 protein and would therefore provide greater sensitivity. The results are shown in Figure 2A.

A deletion mutant lacking the N-terminal 74 amino acids (ΔN75) activated transcription as efficiently as the full-length PHO4–CPF1 fusion protein. However, repression of this construct by co-expression with PHO80 was significantly less efficient, only 7-fold, ~5-fold less than that obtained using the full-length PHO4 protein, suggesting that the N-terminal 74 amino acids were required for efficient repression by PHO80 but that additional residues were also involved. In contrast to the ΔN75 deletion, removal of a further 10 amino acids (ΔN85) almost abolished the ability of the PHO4–CPF1 fusion to activate transcription, with activation being ~70-fold less than the full-length protein. This construct retained some degree of repressibility by PHO80 (~4-fold) but it is difficult to say whether this is significant, given the low levels of transcription activation observed (but see below). Further deletion to amino acid 95 (ΔN95), completely removing the potential acid activation domain, abolishes even the residual activity observed using the ΔN85 mutant. Residues between amino acids 74 and 95 are therefore essential for the function of the PHO4 activation domain, with those residues between 74 and 85, predicted to form an acidic amphipathic α-helix, playing a major role.

### *Two regions of PHO4 interact independently with PHO80*

The results presented demonstrate that the requirements for repression by PHO80 are distinct from those for DNA-binding. However, since deletion further than amino acid 94 abolishes the ability of PHO4 to activate transcription it is not possible, using this assay, to ask which regions of PHO4 are required for repression and in particular whether the PHO4 activation domain itself participates in interaction with PHO80. We therefore made use of the two-hybrid system described in Figure 1 to determine the requirements for interaction of PHO4 with PHO80. Using this system regions of PHO4 able to interact with PHO80 can be identified irrespective of whether the PHO4 activation domain is intact. Initially, a series of N-terminal deletion mutants of PHO4 was constructed, tagged with the VP16 activation domain and then co-expressed with the LexA–PHO80 fusion protein. Transcription was then measured from the *lexA* operator *CYC–lacZ* reporter. As a control for the production of functional protein, the series of mutants was also assayed for their ability to activate transcription from the *PHO5* UAS. The results from a typical experiment are shown in Figure 3.

As before, co-expression of full-length PHO4–VP16 with LexA–PHO80 strongly activates transcription from the *lexA* operator *CYC–lacZ* reporter. Similar levels of activation are obtained using mutants ΔN31 and ΔN75 which lack N-terminal amino acids but retain intact the PHO4 activation domain. Surprisingly, mutant ΔN85, which is severely impaired in its ability to activate transcription, and mutant ΔN95, which lacks the entire PHO4 activation domain and is transcriptionally inactive (Figure 2), both retain the ability to activate transcription through interaction with the LexA–PHO80 chimera. Thus, unlike repression of GAL4 by GAL80 or p53 by MDM2 where the activation domain itself interacts with the repressor, the PHO4 activation domain is not required for interaction with PHO80. Indeed, ΔN156, which lacks the N-terminal 155 residues, retains the ability to interact with PHO80. In contrast, mutants ΔN200 and ΔN218 fail to interact significantly with the LexA–PHO80 fusion, activating transcription from the *lexA* operator ~100-fold less well than the full-length PHO4–VP16 protein. As a control for the production of functional protein, the entire series of N-terminal deletion mutants was also assayed for their ability to activate the *PHO5* UAS, with no more than a 2- to 3-fold variation in activation being observed (not shown). These data therefore point to a region of PHO4 between amino acids 156 and 200 as being a principal site for interaction with PHO80.

Although amino acids 156–200 confer the ability to interact with PHO80, previous data (Figure 2A) suggested a possible role for the N-terminal 74 amino acids, since deletion of these residues resulted in decreased repressibility by PHO80. To determine whether the N-terminus of PHO4 could also mediate interaction with the PHO80 repressor, we constructed and expressed a series of VP16-tagged internal in-frame deletions which removed successively larger portions of the PHO4 protein N-terminal to an engineered *Xho*I site located immediately adjacent to the PHO4 bHLH domain. The results obtained are shown in Figure 4. As expected internal deletions removing residues between positions 200 and the bHLH domain (Δ218int and Δ200int) had no effect on the ability of these proteins to activate via interaction with PHO80, activating transcription efficiently from the *lexA* operator when co-expressed with the LexA–PHO80 chimera. Surprisingly, a similar level of activation, comparable to that obtained using the full-length PHO4–VP16 chimera, was also obtained using mutant

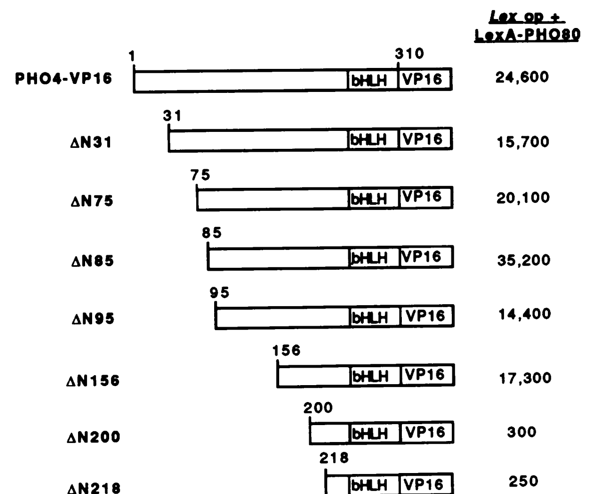



**Fig. 3.** PHO4 amino acids 156–200 mediate interaction with PHO80. The indicated PHO4 N-terminal deletion mutants fused to the VP16 activation domain were expressed together with the LexA–PHO80 chimera, and activation from the *lexA* operator *CYC–lacZ* reporter measured. The levels of β-galactosidase activity shown are given to the nearest 50 units. In control experiments (not shown), all the deletion mutants, including ΔN200 and ΔN218, were able to activate the *PHO5* UAS to approximately similar levels indicating that similar levels of protein were being expressed. The results presented are those from a single experiment. In other experiments up to 2-fold variations in β-galactosidase activities were also apparent but these variations showed no specific pattern and were regarded as not significant and resulting from experimental variation.

Δ156int. Since this mutant lacks the region of PHO4 between amino acids 156 and 200 which are essential for interaction with PHO80 in the context of the N-terminal deletions, we must conclude that a second region of PHO4, N-terminal to amino acid 156, can also participate in interactions with PHO4. Mutants Δ118int and Δ94int, which retain the PHO4 activation domain, also interact with PHO80, while similar levels of activation, and therefore transcription, are obtained using mutants Δ74int and Δ31int, which lack the PHO4 activation domain. These results confirm that the PHO4 activation domain plays no role in interaction with PHO80

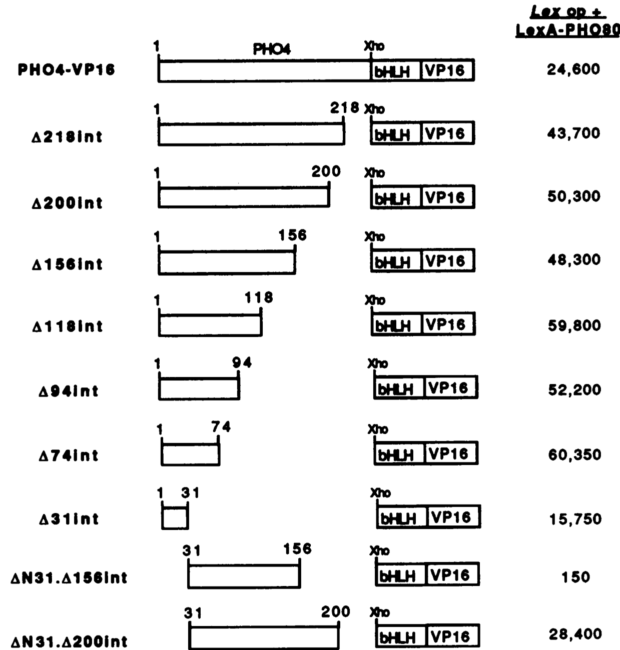



**Fig. 4.** The N-terminal 31 amino acids of PHO4 can independently interact with PHO80. The indicated internal deletions of the PHO4–VP16 chimera were co-expressed with the LexA–PHO80 fusion protein and activation from the *lexA* operator was measured. All internal deletions extended towards the N-terminus from an engineered *Xho*I site located immediately N-terminal to the PHO4 basic region. The levels of β-galactosidase activity shown are given to the nearest 50 units.

and indicate that in addition to amino acids 156–200, the N-terminal 31 amino acids of PHO4 are sufficient to mediate complex formation with the PHO80 repressor. This was confirmed using two additional mutants, ΔN31.Δ200int and ΔN31.Δ156int. Mutant ΔN31.Δ156int lacks both N-terminal and internal interaction domains and as such fails to activate the *lexA* operator *CYC–lacZ* reporter if co-expressed with LexA–PHO80. In contrast, mutant ΔN31.Δ200int, which lacks the N-terminal 31 amino acid region but retains amino acids 156–200, can still recognize the LexA–PHO80 chimera. Thus, both the N-terminal 31 amino acids and the region between amino acids 156 and 200 can independently mediate complex formation between PHO4 and PHO80. We refer to the two regions involved in interaction with PHO80 as repression domains (RD) 1 and 2, RD1 corresponding to amino acids 1–31 and RD2 to residues 156–200.

The importance of RD1 and RD2 for transcription repression was confirmed using a LexA–PHO4 construct. Co-expression of PHO80 repressed LexA–PHO4 by up to 80-fold while repression of LexA–PHO4.ΔN31 or LexA–PHO4.Δ156int was reduced by between 4- and 7-fold (data not shown), confirming the requirements of RD1 and RD2 for efficient repression. Similar results (Figure 5) were obtained by comparing the levels of activation achieved under high or low phosphate conditions using the LexA–PHO4 construct expressed in a strain (Y704) containing endogenous PHO80, or a strain (Y780) in which the *PHO80* gene had been disrupted. In the Y780 strain little significant variation in the levels of activation obtained were observed using either the WT LexA–PHO4 protein or the two derivatives, LexA–PHO4.ΔN31 or LexA–PHO4.Δ156int, lacking the PHO80-interacting domains RD1 and RD2 respectively, irrespective of whether the yeast was cultured under high or low phosphate conditions. In contrast, in the strain Y704, which expresses endogenous PHO80, activation by the WT LexA–PHO4 chimera was repressed 5-fold under high phosphate conditions. Significantly, no repression by high phosphate was observed using the LexA–PHO4.Δ156int mutant, which lacks RD2, while repression of the LexA–PHO4.ΔN31 mutant, lacking RD1, was reduced. Thus, consistent with their abilities to mediate interaction with PHO80, both RD1 and RD2 are required

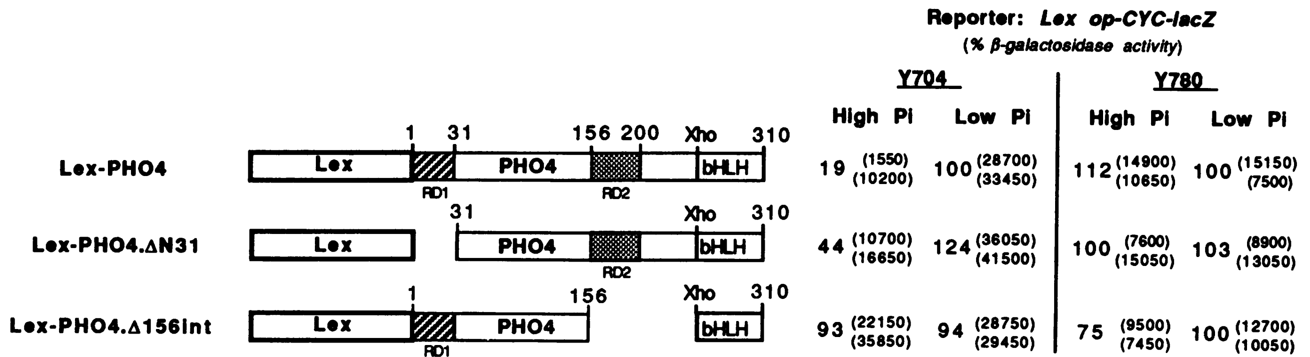



**Fig. 5.** The PHO80 interacting regions, RD1 and RD2, are required for phosphate-mediated repression of PHO4. The LexA–PHO4 fusion and its mutant derivatives were transformed into strain Y704, which expresses endogenous PHO80, or strain Y780, which contains a PHO80 disruption. Yeast was grown under high or low phosphate conditions and the levels of β-galactosidase obtained from a *lexA* operator *CYC–lacZ* reporter were determined. The values given are relative to the activity of WT *lexA–PHO4* under low phosphate conditions (100%), to enable the relative expression in the two different strains to be evaluated. The figures in brackets represent the actual values for β-galactosidase activity obtained from two different experiments.

for efficient repression of DNA-bound PHO4 by PHO80 under high phosphate conditions.

## Discussion

### Repression of the PHO4 transcription activation domain

With multiple factors each able to bind the same sequence present in a cell, the necessity for regulating their potential to activate transcription is evident. Multiple mechanisms may operate, but one of the most important is likely to involve the masking of an activation domain by protein–protein interaction. Yet, to date, with the exception of the repression of GAL4 by GAL80 (Ma and Ptashne, 1987; Salmeron *et al.*, 1990; Leuther *et al.*, 1992) and, more recently, repression of p53 by MDM2 (Oliner *et al.*, 1993), there has been little progress in identifying either the repressors or their targets.

Although the physical association between PHO4 and PHO80 has long been postulated, multiple alternative mechanisms of transcription repression could not be excluded. Indeed, it was only recently, with the characterization of PHO80 mutants that could compensate for the *PHO4* constitutive (*PHO4*$^{c}$) mutation (Okada and Toh-e, 1992), that evidence suggesting a direct interaction between the two proteins was obtained. Biochemical studies on the mechanism of repression of PHO4 by PHO80 have been frustrated by an inability to express PHO80 protein in *Escherichia coli* in sufficient quantities for *in vitro* analysis. In an alternative approach using the two-hybrid system, we have been able to provide conclusive evidence that repression of PHO4 by PHO80 involves an association between the two proteins, the consequence of which is inactivation of the PHO4 activation domain rather than inhibition of DNA-binding. Our data are therefore consistent with the model in which under high phosphate conditions PHO4 can bind DNA as a complex with PHO80. Presumably interaction with PHO80 either directly prevents the PHO4 activation domain interacting with components of the basal transcription machinery or induces a conformation change in PHO4 incompatible with transcription activation. As phosphate levels are reduced the PHO4 activation domain is unmasked either following dissociation of PHO80 from PHO4 or as a consequence of a conformational change in the PHO4–PHO80 complex.

Two regions of PHO4 were identified, each independently able to mediate interaction with PHO80. The relative locations of the activation domain, T, and the region required for interaction with PHO80 are summarized in Figure 6. Region RD1, between residues 1 and 3, lies N-terminal to the transcription activation domain. Deletion of RD1 does not inhibit the ability of the PHO4–CPF1 chimera to activate transcription and reduces, but does not abolish, the ability of PHO80 to repress. A second region, RD2, between residues 156 and 200, can also bind PHO80, consistent with the observation that mutations at, or close to, residue 174, within RD2, induce the PHO4$^{c}$ phenotype (Ogawa and Oshima, 1990). However, it should be noted that transcription activation by *PHO4*$^{c}$ mutants under high phosphate (repressing) conditions remains ~10-fold less than levels obtained with a WT PHO4 protein placed under low phosphate (inducing) conditions (Ogawa and Oshima, 1990). This strongly suggests that the *PHO4*$^{c}$ mutations previously described are not by themselves sufficient to disrupt the

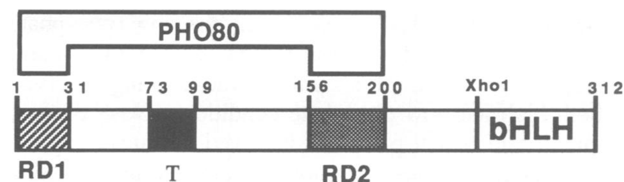



**Fig. 6.** Schematic diagram showing the relative locations of the PHO4 transcription activation domain, T, and the two regions RD1 and RD2, that can each independently mediate interaction with PHO80. Repression by PHO80 in the model shown is indicated by two regions of a single molecule of PHO80 interacting with RD1 and RD2. Although there is no apparent amino acid sequence homology between RD1 and RD2 it is also possible that each site of interaction is recognized by a different molecule of PHO80.

PHO4–PHO80 complex, most likely because the independent association of PHO80 with RD1 is maintained. These data, together with the observation that deletion of RD1 or RD2 reduces repression by PHO80 of a LexA–PHO4 fusion, confirm that the interaction between RD1 and RD2 with PHO80 detected using the two-hybrid system is relevant for repression of DNA-bound PHO4, and is relevant for regulation of PHO4 by phosphate.

Although repression of PHO4 by PHO80 superficially resembles that of GAL4 by GAL80 in that repression arises from the transcription activation domain being masked, the mechanisms underlying repression are clearly different. Essential residues of the PHO4 activation domain are located between amino acids 75 and 99; transfer of this region to the heterologous GAL4 DNA-binding domain efficiently activates transcription from a *GAL* UAS (our unpublished observations) while removal of the N-terminal 74 amino acids in the PHO4–CPF1 chimera did not reduce its ability to activate transcription. Deletion of the PHO4 activation domain did not diminish the ability of the mutant protein to interact with PHO80, demonstrating that requirements for transactivation and complex formation are clearly distinct and separable. Indeed, RD2 is located some 60 amino acids C-terminal to the activation domain. In contrast, the requirements for repression by GAL80 and transcription interaction map to the same region of GAL4 (Ma and Ptashne, 1987; Leuther *et al.*, 1990; Salmeron *et al.*, 1990). Thus, while the GAL4 activation domain is masked directly by interaction with GAL80, the PHO4 activation domain is masked indirectly, as a consequence of PHO80 interaction with regions outside the activation domain.

A recent report, using an activation domain-tagged GAL80, suggested that GAL4 and GAL80 remain associated *in vivo* after galactose induction (Leuther and Johnston, 1992), implying that activation is a consequence of a conformational change in the complex rather than of the GAL4 activation domain becoming exposed after dissociation of GAL80. The consequences of phosphate induction for the PHO4–PHO80 complex are less apparent: we have tried unsuccessfully to detect differential association under high and low phosphate conditions of PHO4–VP16 with LexA–PHO80 in our two-hybrid system. This may mean that the two proteins remain associated under de-repressing, low phosphate conditions. However, we believe that this conclusion would be premature; activation by PHO4 is inefficient in low phosphate medium compared with that obtained in a PHO80 disruption strain (our unpublished observations), and as such a proportion of the tagged PHO80 protein is likely to remain

associated with PHO4 even under the low phosphate conditions used to de-repress PHO4. In the two-hybrid system this would be reflected in activation being observed in both high and low phosphate conditions. Nevertheless, despite the technical problems involved in elucidating the events underlying de-repression, it remains an intriguing possibility that PHO80 stays associated with at least one of the interacting domains as PHO4 activates its target genes.

Whether each molecule of PHO4 interacts with more than one molecule of PHO80 is also unclear. However, given the fact that RD1 and RD2 exhibit no obvious sequence homology we favour the idea that RD1 and RD2 each interact with different regions of a single PHO80 molecule (see Figure 5) and we are currently using the two-hybrid system to investigate this possibility. The characterization of the precise mechanism that modulates the PHO4–PHO80 complex to allow transcription activation must therefore await the development of an efficient *in vitro* system.

### *A paradigm for transcription regulation*

Repression of PHO4 by PHO80 represents a highly efficient means of controlling gene expression, and it is entirely possible that similar regulatory mechanisms operate to regulate the activation potential of other transcription factors. In this respect, the parallels between PHO4–PHO80 interaction and negative regulation of the c-Jun cell-type-specific activator region, a1, are striking (Baichwal *et al.*, 1992). Mutants in a1 exhibit reduced potential to activate transcription but retain the ability to interact with a repressor. Repression requires two separate domains, $\delta$ and $\epsilon$; the $\delta$ region lies N-terminal to the a1 activation domain and is required for full repression, while mutants that lack $\epsilon$, located C-terminally to a1, fail to bind the repressor and are constitutively active. Thus, repression appears to be mediated by interaction with $\epsilon$ and $\delta$ with the activation domain a1 being masked as a consequence. The $\delta$, a1 and $\epsilon$ regions of c-Jun are therefore highly reminiscent of RD1, T and RD2 of PHO4, although whether this similarity is only superficial or whether c-Jun and PHO4 share a common regulatory mechanism will only be apparent when the c-Jun repressor is isolated.

For the bHLH family of transcription factors, the PHO4–PHO80 interaction may represent the first characterized example of differential protein–protein interaction designed to target a transactivation domain rather than the capacity to bind DNA. Moreover, the similarities with c-Jun might represent evidence that the PHO4–PHO80 interaction may be taken as a paradigm for regulation of a wide variety of transcription factors by an evolutionarily conserved mechanism. Given the large number of transcription factors operating with an individual cell, it seems likely that regulating the activating potential of transcription factors will represent a common regulatory mechanism. Support for this idea comes from the recent observation that the product of the *MDM2* gene conceals the activation domain of the p53 tumour suppressor protein (Oliner *et al.*, 1993). Indeed, the use of an activator–repressor complex that targets the activation domain rather than DNA-binding has a number of advantages. Since the repressor–activator complex retains the abilty to bind DNA, once bound, it may act as a sequence-specific repressor, preventing access by other factors sharing the same DNA-binding specificity. In addition, the ability to recognize a specific site in the repressed state allows an instantaneous transcriptional response to signals mediating de-repression, without the need either to find its target sites and perhaps displace other factors, or for the additional protein–protein interactions necessary if DNA-binding was inhibited by differential heterodimerization.

It is clear that further characterization of the mechanism by which PHO80 repressed PHO4 will prove extremely valuable for understanding the molecular mechanisms underlying regulation of eukaryotic gene expression.

## Materials and methods

### *Yeast strains and media*

*Saccharomyces cerevisiae* strain Y704 ($\alpha$, *ade2*-1, *trp1*-1, *can1*-100, *leu2*-3, *leu2*-112, *his3*-11,15, *ura3*, *pho4:HIS3*) (Fisher *et al.*, 1991) was used for all assays except for Figure 5, where strain Y780 ($\alpha$, *ade2*-1, *trp1*-1, *can1*-100, *leu2*-3, *leu2*-112, *his3*-11,15, *ura3*, *pho80:HIS3*) was also used. Yeast cultures were grown at 30°C in either YPD (1% yeast extract, 2% glucose, 1% peptone) or minimal medium (0.67% yeast nitrogen base, 1% glucose) supplemented with the appropriate amino acids (0.002%) as required except for the experiment shown in Figure 5 which involved growth in low (0.1 mM) or high (16 mM) phosphate medium.

### *Yeast transformations and β-galactosidase assays*

Yeast transformations were performed following the procedure of Hinnen *et al.* (1978). For 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 and resuspended in 1 ml minimal medium, and 100 μl was transferred into 6 ml galactose minimal medium to induce expression of proteins under the control of the *GAL10* promoter. For the experiments involving phosphate regulation, 100 μl of yeast from the glucose minimal culture were transferred to 6 ml low or high phosphate galactose minimal medium containing 1.6% glucose. The glucose was present to reduce expression from the *GAL* promoter and to enable lower level expression of the LexA–PHO4 chimeras so that regulation by phosphate could be observed. 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, 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 multiple times.

### *Yeast vectors*

The *PHO5* UAS *CYC–lacZ* reporter has been described previously (Fisher *et al.*, 1991). The reporter containing the *lexA* operator was constructed by insertion of three copies of a double-stranded oligonucleotide (5′-ggatccagatctTACTGTATGTACATACAGTACAGACTACTGTATGTACATACAGTACggatcc-3′) into the unique *Bgl*II site upstream of the *CYC–lacZ* reporter in p669B (Cousens *et al.*, 1989). The 80 amino acid VP16 activation domain was isolated by PCR using the following primers: 5′-agacggatccagatctTCGACGGCCCCCCCGACCGAT-3′ and 5′-agaggatccTAGTTAGTCACCCACCGTACTCGTCAATTCC-3′ and cloned into the *Bam*HI site of pTZ (Pharmacia). PHO4 WT and deletion mutants were isolated by PCR using the appropriate primers and inserted as *Bam*HI fragments into the unique, engineered *Bgl*II site located at the N-terminus of the VP16 activation domain PCR product. In-frame fusion proteins were then inserted as *Bam*HI fragments into the yeast expression vector pKV701 (*LEU*) (Fisher *et al.*, 1991) under the control of the inducible *GAL10* promoter. All deletion mutants were verified by sequencing.

The PHO80 coding sequence was isolated by PCR as a *Bam*HI fragment from yeast genomic DNA and cloned into pUC18 and the sequence verified and subsequently transferred into the *Bgl*II sites of yeast expression vectors with appropriate selectable markers. For repression of the PHO4 derivatives expressed from the pKV701 (*LEU*) vector, PHO80 was expressed from pRS424.KV(*TRP*), containing the *GAL10* promoter and PGK terminator. The LexA expression vector 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. The PHO80 coding sequences were inserted as an *Xho*I PCR product in-frame with *lexA* and upstream of the *CYC* terminator sequence.

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## Note added in proof

Recent evidence indicates that PHO80 can act as a cyclin, targeting the PHO85 cyclin-dependent kinase to PHO4 and resulting in its phosphorylation [Kaffaman *et al.* (1994) *Science*, **263**, 1153–1156). We have also shown (F.Fisher, P.C.McAndrew, K.Hirst and C.R.Goding, submitted) that PHO4 interacts co-operatively with the homeobox protein PHO2 in low but not high phosphate. Thus, while PHO80 regulates PHO4 directly by masking its activation domain, the PHO80–85 complex also appears to regulate PHO4 DNA binding through its interaction with PHO2.