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# The homeodomain protein Pho2 and the basic-helix–loop–helix protein Pho4 bind DNA cooperatively at the yeast *PHO5* promoter

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## ABSTRACT

**Two transcription factors, the bHLH protein Pho4 and the homeodomain protein Pho2, are required for transcriptional activation of the *PHO5* promoter in *Saccharomyces cerevisiae*. There are two essential Pho4 binding sites, corresponding to the regulatory elements UASp1 and UASp2 at the *PHO5* promoter, but only a single, dispensable Pho2 binding site had previously been identified. We have reinvestigated binding of Pho2 to the *PHO5* promoter using purified recombinant protein and have found multiple Pho2 binding sites of different affinities along the promoter. One of the high affinity Pho2 sites largely overlaps the Pho4 binding site at UASp1. Cooperative DNA binding of the two proteins to their overlapping sites, resulting in a high-affinity ternary complex, was demonstrated. Pho2 and Pho4 also bind DNA cooperatively at UASp2 where two Pho2 sites flank the Pho4 site. Finally, Pho2 facilitates binding of Pho4 to a third, cryptic Pho4 binding site which binds Pho4 with lower affinity than UASp1 or UASp2. These results suggest that cooperative DNA binding with Pho4 is integral to the mechanism by which Pho2 regulates transcription of the *PHO5* gene.**

## INTRODUCTION

When the yeast *Saccharomyces cerevisiae* is grown under conditions of phosphate limitation, production of acid phosphatase is increased dramatically as a result of transcriptional activation of the *PHO5* gene (1). Genetic studies of the *PHO5* system revealed that two transcription factors, the basic-helix–loop–helix protein Pho4 and the homeodomain protein Pho2, are required for the induction of the *PHO5* promoter (2). The activity of Pho4 is regulated in response to phosphate levels through phosphorylation by a cyclin–CDK complex, encoded by *PHO80* and *PHO85*, respectively. When phosphate is abundant, Pho4 is inactivated by phosphorylation, while upon phosphate starvation, the positive factor Pho81 prevents phosphorylation of Pho4 by inhibiting the Pho80–Pho85 complex (3). Recent data suggest that phosphorylation of Pho4 affects its subcellular localization with the phosphorylated form being localized in the cytoplasm and thereby unable to activate *PHO5* transcription (4).

Deletion analysis of the *PHO5* promoter demarcated two regulatory elements, UASp1 and UASp2 (5), to which Pho4 has been shown to bind *in vitro* (6). *In vivo* footprinting experiments revealed that Pho4 binds to both sites upon phosphate starvation, but not at high phosphate conditions (7). In addition, Pho4 has been shown to bind to promoters of other genes which are regulated by phosphate, including *PHO8* (8), *PHO10* and *PHO11* (9), *PHO81* (10) and *PHO84* (11).

In contrast with the clear picture for Pho4, the role of Pho2, the other activator of *PHO5* regulation, has proved more difficult to define. Pho2 is a homeodomain protein which has been shown to regulate a diverse array of genes, including not only *PHO5*, but also the *HO* gene (12), the *HIS4* (13), *TRP4* (14) and the *ADE1*, *ADE2*, *ADE5,7* and *ADE8* genes (15). In each case, a Pho2 binding site has been demonstrated in the promoter region *in vitro*. In the *PHO5* promoter, a single Pho2 binding site was mapped between the two Pho4 binding sites by *in vitro* footprinting (6). However, the role of Pho2 in *PHO5* regulation has remained enigmatic, because deletion of the Pho2 binding site did not influence *PHO5* promoter activity significantly (5). In addition, activation of a heterologous promoter by a 31 bp sequence containing UASp1 is fully Pho2-dependent, even though there was no evidence for Pho2 binding to this element (16). Finally, although Pho2 is required for *PHO5* promoter activation, overexpression of Pho4 can give rise to a limited activation of the *PHO5* promoter in the absence of Pho2 (17).

For some of the Pho2-dependent genes, there are indications of a role for Pho2 in interacting with other transcription factors. At the *HO* promoter, a Pho2 binding site is located next to a Swi5 binding site, and it was shown that the two proteins bind to their sites cooperatively (12). In the case of the *HIS4* promoter, a Pho2 (Bas2) protected region largely overlaps the Bas1 footprint. Although Pho2 and Bas1 can bind to this region simultaneously, no cooperative interactions between the two proteins were detected (13). In contrast, at the *TRP4* promoter, a Pho2 binding site completely overlaps one of the two Gcn4 binding sites, and the two proteins were found to bind DNA in a mutually exclusive manner (14). Recent experiments have demonstrated an interaction between Pho2 and a specific domain of Pho4 using the yeast two hybrid system (18), raising the possibility of an additional role of Pho2 also at the *PHO5* promoter.

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In an attempt to resolve some of the questions concerning the mechanism by which Pho2 contributes to *PHO5* regulation, we have reinvestigated Pho2 binding at the *PHO5* promoter and examined the possibility of Pho2–Pho4 interactions. Our results show that there are multiple Pho2 binding sites at the *PHO5* promoter. One of them significantly overlaps with the Pho4 binding site at UASp1, while another two sites flank the second Pho4 binding site, UASp2. In addition, a cryptic Pho4 binding site has been mapped 60 bp downstream of UASp2 with a Pho2 binding site at its 3′ side. Binding studies reveal that Pho2 binds cooperatively with Pho4 at each Pho4 binding site, providing a mechanism that explains the absolute requirement of Pho2 for *PHO5* promoter activation.

## MATERIALS AND METHODS

### Plasmids

The *PHO2-HIS* expression plasmid has been described previously (19). The *PHO4-HIS* expression plasmid was created from a plasmid in which an *Nco*I site had been inserted at the *PHO4* start codon (provided by A. Hinnen). An *Nco*I–*Bbr*PI fragment of this plasmid was cloned into *Nco*I–*Eag*I-digested pET-21d (Novagen). This adds 10 amino acids to the C-terminus of Pho4, the last six of which are the histidine tag.

*PHO5* DNA restriction fragments used for DNase I footprinting and gel shift analyses were derived from *PHO5–lacZ* fusions (20) containing the wild-type promoter as well as from subcloned derivatives providing restriction sites not present in the wild-type promoter.

### Expression and purification of Pho4-HIS and Pho2-HIS fusion proteins

Purification of the Pho2-HIS fusion protein has been described previously (19,21). Pho4-HIS protein was similarly purified using Qiagen $Ni^{2+}$–NTA–agarose except that the column was washed with buffer containing 60 mM imidazole prior to eluting with buffer containing 1 M imidazole. Peak fractions containing Pho4-HIS or Pho2-HIS were pooled and dialyzed against 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.5 mM EDTA, 0.2 mM PMSF, 1 mM benzamidine. As judged by SDS–gel electrophoresis, both proteins were highly purified. A monoclonal antibody against the histidine tag was obtained from Dianova, Hamburg, Germany.

### DNase I footprinting

Purified Pho2-HIS and/or Pho4-HIS were incubated with labeled DNA fragments (~15 000 c.p.m.) for 30 min at room temperature in 25 µl of 10 mM Tris–HCl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and 10 µg/ml poly(dI–dC). After the incubation, 25 µl of 1 U/ml DNase I, diluted in the same reaction buffer containing in addition 1 mM $CaCl_2$, was added and the reaction was incubated at room temperature for 1–2 min. The reaction was stopped by addition of 50 µl of stop solution containing 0.4 M NaCl, 0.4% SDS and 25 µg/ml of salmon sperm DNA. The DNA was precipitated with ethanol and analyzed on 6 or 8% polyacrylamide–8 M urea gels.

### Gel shift assays

DNA fragments were labeled with [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) using polynucleotide kinase, and ~5000 c.p.m. of labeled DNA was used per binding reaction. Protein–DNA binding reactions (10 µl) were performed in 15 mM Tris–HCl, pH 8.0, 75 mM NaCl, 7.5% glycerol, 12.5 mM DTT, 0.375 mM EDTA, 750 µg/ml bovine serum albumin and 25 µg/ml poly(dI–dC). The amount of protein used in each binding reaction is indicated in arbitrary units in the figure legends. One unit of Pho4 and Pho2 corresponds to ~5 and 6 ng protein, respectively, as determined by SDS–gel electrophoresis. Reactions were incubated at room temperature for 30–45 min before they were loaded onto a 5% polyacrylamide gel made in TBE buffer (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 8.3). Electrophoresis was in the same buffer for ~1.5 h at 250 V, then the gel was dried and autoradiographed with an intensifying screen.

## RESULTS

### Multiple Pho2 binding sites are found at the *PHO5* promoter

In order to look for additional Pho2 targets in the *PHO5* promoter, we have reinvestigated binding of Pho2 which was purified by the Hexa-HIS-tag method. DNA fragments encompassing the two Pho4 binding sites, UASp1 and UASp2, were examined by *in vitro* DNase I footprinting. As shown in Figure 1, several protected regions are seen on both the upper and the lower strand in the presence of added Pho2. Two footprints, separated by a short unprotected region, are found between UASp1 and UASp2 from –271 to –285 and from –291 to –320 (Fig. 1A). This area contains the previously determined Pho2 binding site (–277 to –296) (6). In addition to these two regions, Pho2 protection is observed from –358 to –385 (Fig. 1B), i.e. over a sequence partially overlapping UASp1. A stepwise decrease in the Pho2 concentration results in the simultaneous loss of Pho2 protection at all three regions, suggesting similar affinities of Pho2 for these binding sites (Fig. 1A and B).

Pho2 protection (and enhancement) is also observed upstream of UASp1 as well as downstream of UASp2. To map these sites more precisely, suitable DNA fragments were examined. As shown in Figure 1C, the region upstream of UASp1 contains multiple closely adjacent sites protected by Pho2, in addition to the one partially overlapping UASp1. Two sites are found downstream of UASp2 from –223 to –236 and –169 to –182 and an additional protected area around –110 (Fig. 1D).

As summarized below in Figure 9, Pho2 can bind with different affinities (see below) to multiple sites between the TATA box and the upstream *Bam*HI site. One of the strong Pho2 binding sites partly overlaps the previously determined Pho4 binding site at UASp1, which is consistent with a recent report that Pho2 from a yeast extract can bind to an oligonucleotide containing the UASp1 sequence (22). In addition, there are two Pho2 binding sites that flank UASp2. It was of interest, therefore, to determine if Pho4 and Pho2 can simultaneously bind to UASp1 as well as to UASp2, and if binding of one protein influences binding of the other.

### Pho2 and Pho4 bind cooperatively to their overlapping sites at UASp1

We first examined binding of Pho2 and Pho4 to a DNA fragment containing UASp1 (Fig. 2). The two proteins appear to be able to bind simultaneously to UASp1, even though their individual footprints overlap significantly. Eleven out of 19 nucleotides

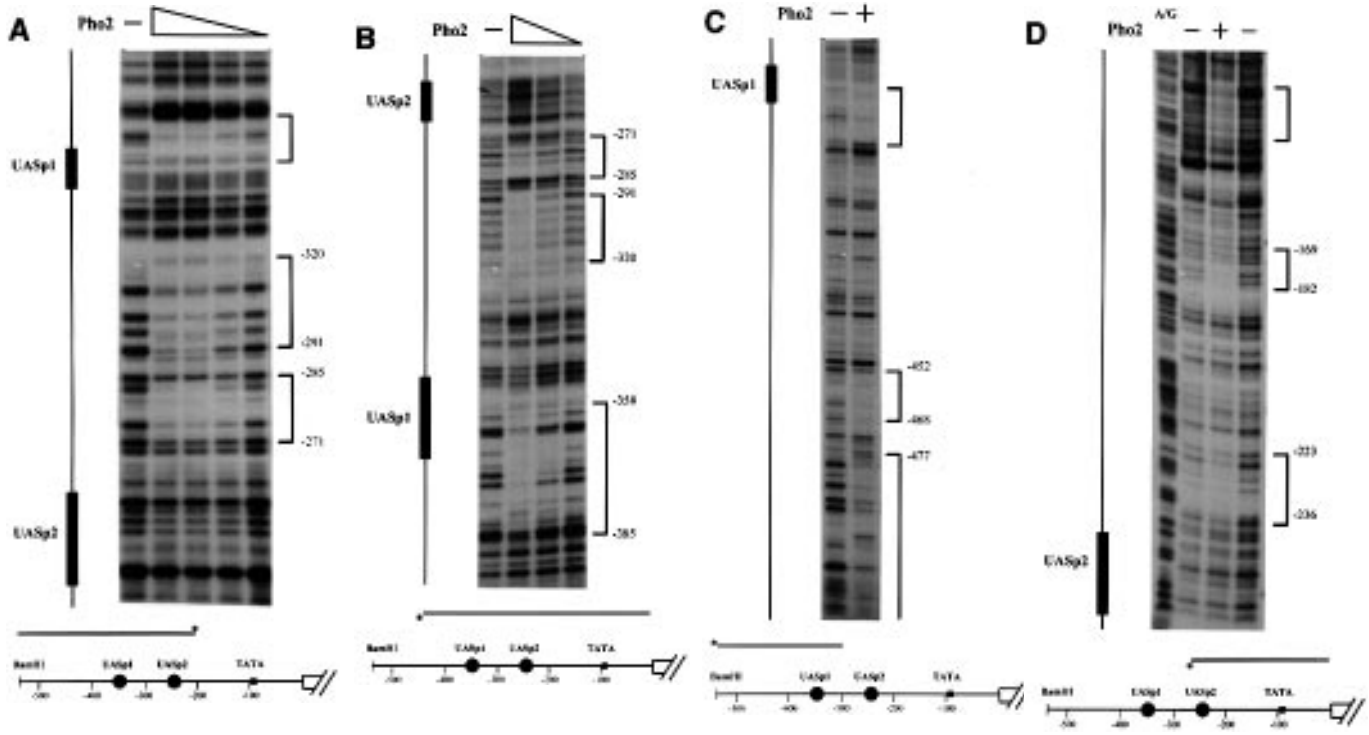


**Figure 1.** DNase I footprint analysis of the *PHO5* promoter with Pho2. DNase I footprinting was performed as described in Materials and Methods. The upper strand of an *Sfu*I (–206) *Bam*HI (–542) fragment was labeled at the *Sfu*I site (**A**), the lower strand of a *Bsa*HI (–444) *Bam*HI (+9) fragment at the *Bsa*HI site (**B**), the upper strand of a *Bam*HI (–542) *Eco*RI (–324) at the *Bam*HI site (**C**), and the lower strand of a *Hin*dIII (–287) *Bam*HI (+9) at the *Hin*dIII site. All fragments are derived from *PHO5–lacZ* derivatives (see Materials and Methods). The fragments are schematically indicated relative to the promoter at the bottom and regions protected by Pho2 are marked on the right. In (C) there is additional enhancement and protection of DNase I cleavage sites not marked in the figure. The –291 to –320 and the –358 to –385 regions may actually consist of two adjacent or partially overlapping Pho2 binding sites. A partial purine specific degradation pattern on the left serves as a marker in (D).

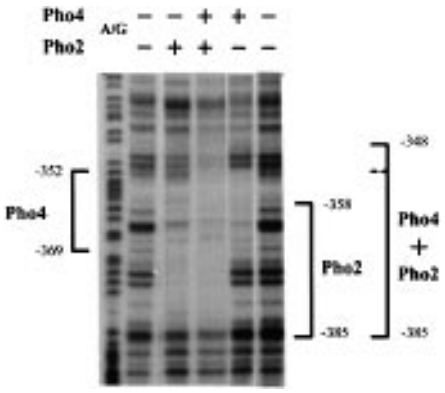


**Figure 2.** Pho2 and Pho4 can simultaneously bind to overlapping sites at UASp1. DNase I footprinting was performed as described in Materials and Methods. The lower strand of a *Bsa*HI (–444) *Bam*HI (+9) fragment was labeled at the *Bsa*HI site. Pho4 and Pho2 were added separately or together as indicated at the top. A partial purine specific degradation pattern on the left serves as a marker. The protected regions are indicated on the side.

protected by Pho4 on the bottom strand are also protected by Pho2. The protection pattern found with both proteins is larger by a few nucleotides in the downstream direction than the sum of the individual patterns.

The possibility of cooperative DNA binding by Pho2 and Pho4 was investigated by performing gel shift experiments. A PCR generated fragment, containing UASp1 and the overlapping Pho2 site, was incubated with increasing amounts of Pho4 in the absence or presence of a constant amount of Pho2 (Fig. 3, lanes 1–9). Conversely, the amount of Pho2 was varied in the absence or presence of a constant amount of Pho4 (lanes 10–18). Adding Pho4 and Pho2 together to the DNA yields a more slowly migrating protein–DNA complex (lanes 6–9 and 15–18). Because the footprint data show that both proteins can bind simultaneously to this DNA fragment (Fig. 2), we conclude that the lower mobility complex represents a ternary Pho2–Pho4–DNA complex. All bands in these gel shifts can be supershifted with a HIS-tag antibody (not shown) demonstrating that the complexes arise only from Pho2 and/or Pho4 binding to the DNA. Comparison of Pho4 binding in the absence (lanes 1–4) and presence (lanes 6–9) of Pho2 indicates that the apparent affinity of Pho4 for this DNA fragment increases in the presence of Pho2. This can also be observed in lanes 6–9 where there is a disproportionate increase in the amount of ternary complex relative to the binary complex containing Pho4 alone. Similarly, the addition of Pho4 stimulates binding of Pho2 (compare lanes 10–13 with lanes 15–18).

## Pho2 and Pho4 bind cooperatively to adjacent sites at UASp2

The Pho4 binding site at UASp2 does not overlap with a Pho2 binding site, but Pho2 sites are immediately adjacent (Fig. 4). There are only 2–5 unprotected nucleotides between the Pho4 and downstream Pho2 protected regions, while the upstream Pho2

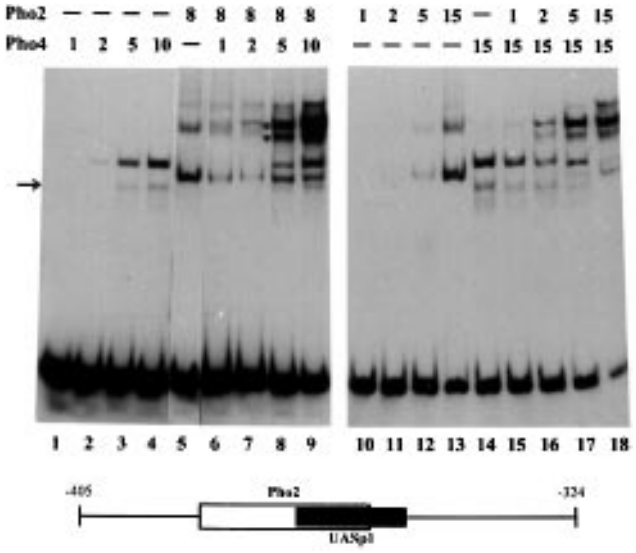


**Figure 3.** Cooperative DNA binding of Pho2 and Pho4 at UASp1. The binding reaction and the gel shift assay were performed as described in Materials and Methods. A labeled 81 bp PCR generated fragment (–324 to –405), containing UASp1 and the overlapping Pho2 site(s) was used as shown schematically at the bottom. Pho4 and Pho2 were added separately or together as indicated at the top. Amount of protein added to an assay mixture is given in arbitrary units (see Materials and Methods). The higher mobiliy protein–DNA complex observed with Pho4 added alone represents proteolytically degraded Pho4 protein bound to DNA (marked by an arrow). The positions of the ternary complexes containing either full length Pho4 or the degradation product of Pho4 protein are indicated by asterisks.

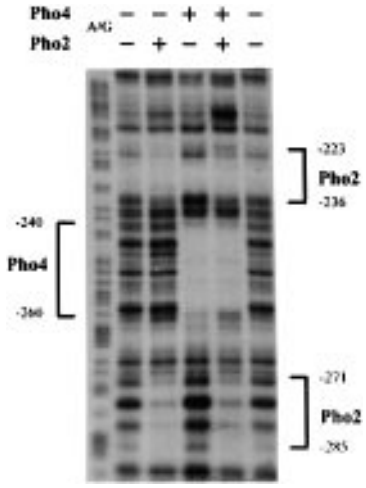


**Figure 4.** Pho2 binding sites are located closely adjacent to the Pho4 site at UASp2. DNase I footprinting was performed as described in Materials and Methods. The lower strand of an *Nco*I (–345) *Bst*EII (–174) fragment was labeled at the *Nco*I site. A partial purine specific degradation pattern on the left serves as a marker. The protected regions are indicated on the side.

footprint is at least 7 nucleotides removed from the Pho4 footprint. None of the footprints are qualitatively altered when both Pho2 and Pho4 are present in the binding reaction.

Cooperative DNA binding of Pho4 and Pho2 to UASp2 was examined by gel shift experiments using a fragment containing UASp2 and the two flanking Pho2 sites. As shown in Figure 5A, binding of Pho4 to UASp2 was enhanced in the presence of Pho2. Furthermore, the presence of Pho4 increases the apparent affinity of Pho2 for its binding sites (not shown). To determine if binding of Pho2 to either of the single sites is sufficient for cooperativity, two additional restriction fragments were analyzed, each containing only one of the two Pho2 sites in addition to UASp2. The upstream Pho2 site appears to bind Pho2 somewhat more strongly than the downstream (compare the ratios of bound versus free DNA in lanes 5 of Fig. 5B and C). However, either site alone can give rise to cooperative DNA binding of Pho4 and Pho2.

There are indications that Pho2 by itself binds cooperatively to the DNA fragment containing both Pho2 sites. Two Pho2–DNA complexes were observed (Fig. 5A, lane 5), and the abundance of the more slowly migrating protein–DNA complex, presumably containing two molecules of Pho2 bound to DNA, is more than would be statistically expected based on the abundance of the more rapidly migrating complex with one Pho2 molecule bound to DNA. The lower mobility complex does not appear to be an oligomeric form of Pho2 bound to a single site, since at a 2-fold higher Pho2 concentration, there was almost no such complex with the fragments containing single Pho2 sites (lane 5, Fig. 5B and C). Therefore, with the DNA fragment containing UASp2 and the two Pho2 sites, what we observe are likely to be cumulative cooperative effects between Pho2 itself and between Pho2 and Pho4.

## DNA binding by Pho2 is required for cooperativity with Pho4

We wished to determine whether protein interactions between Pho2 and Pho4 are sufficient to generate ternary complexes and/or enhance the DNA binding activity of Pho4, and therefore, we examined binding of Pho4 in the absence or presence of Pho2 to a 41 bp restriction fragment from UASp2 containing only the Pho4 site (Fig. 6). This is possible, because, unlike with UASp1, the Pho2 and Pho4 sites do not overlap at UASp2. Pho4 binding to this fragment was identical in the presence and absence of Pho2. No evidence of Pho2 binding was observed, nor was any ternary complex detected (Fig. 6), although even higher Pho2 concentrations were used than in the experiments of Figure 5. These results show that DNA binding by Pho2 is required for the formation of a stable ternary complex and cooperative interactions with Pho4.

## Cooperative DNA binding of Pho2 and Pho4 at a newly mapped weak Pho4 site

As shown in Figure 1D, one region protected by Pho2 is present 70 bp downstream of UASp2 (at position –169 to –182). Adjacent to the Pho2 protected region, there is a sequence -CACATG- which corresponds to the Pho4 consensus binding site (CACGTG) with just one mismatch. We therefore examined binding of Pho2 as well as Pho4 to this region. At lower Pho4 concentrations, which are still sufficient to give clear footprints at UASp1 and UASp2, Pho4 binding is not detectable (not shown). However, at higher Pho4 concentrations there is a footprint from position –190 to –200, which is separated from the adjacent Pho2 footprint by only a few nucleotides (Fig. 7).

In gel shift experiments, Pho4 bound to a fragment containing this region (Fig. 8), albeit with considerably lower affinity than to UASp1 and UASp2 (compare lanes 1–4 in Figs 8, 3 and 5A). Binding of high levels of Pho4 to this site has recently been

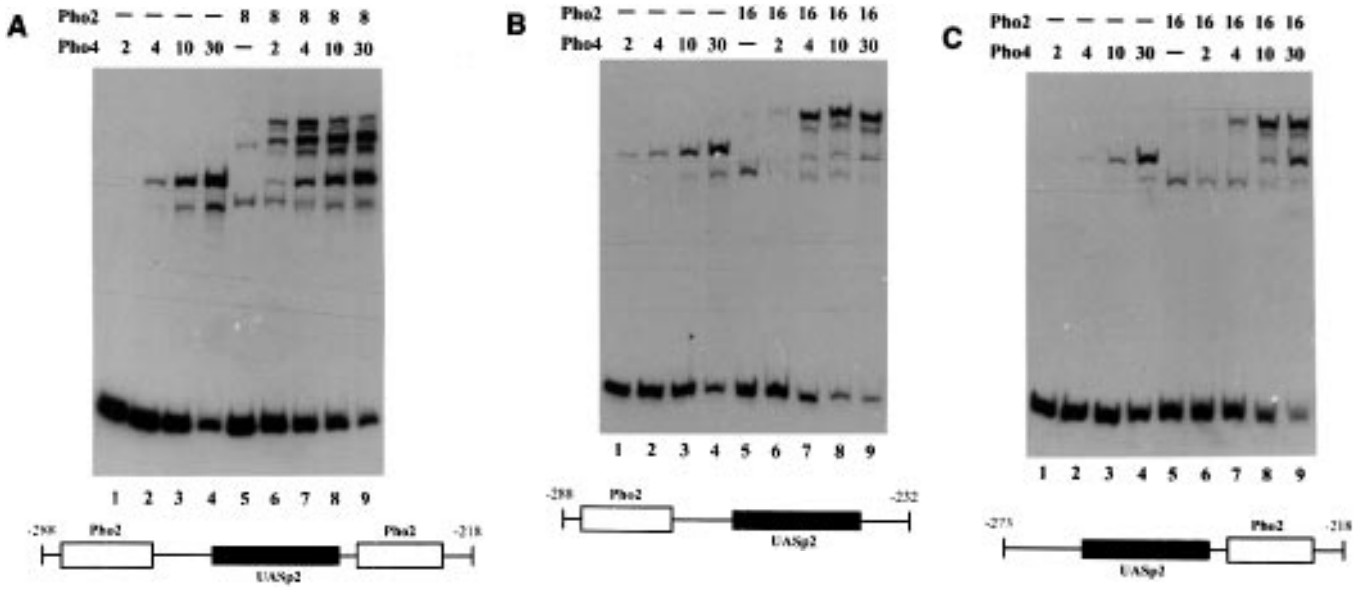


**Figure 5.** Pho2 and Pho4 bind cooperatively to DNA at UASp2. The binding reaction and gel shift assay were performed as described in Materials and Methods. Three different promoter fragments were used as shown schematically at the bottom: (**A**) *Xho*I (–288) *Ava*II (–218) fragment, containing UASp2 and Pho2 sites located upstream and downstream of the Pho4 site; (**B**) *Xho*I (–288) *Apo*I (–232) fragment, containing UASp2 and the upstream Pho2 site; (**C**) *Cla*I (–273) *Ava*II (–218) fragment, containing UASp2 and the downstream Pho2 site.

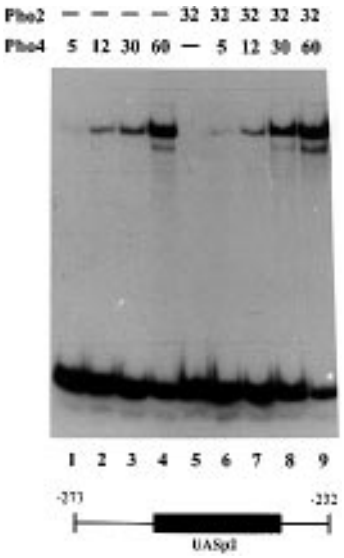


**Figure 6.** Effect of Pho2 on the binding of Pho4 to a fragment containing UASp2 without adjacent Pho2 sites. The binding reaction and gel shift assay were performed as described in Materials and Methods. Binding of Pho4 in the absence or presence of Pho2 to a *Cla*I (–273) *Apo*I (–232) promoter fragment (shown schematically at the bottom), containing UASp2 but no Pho2 sites, was examined.

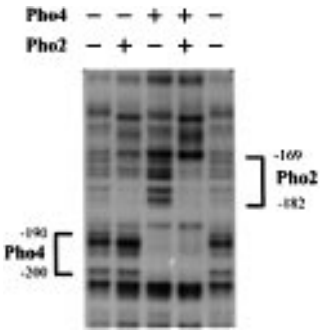


**Figure 7.** A third Pho4 site is mapped downstream of UASp2. The lower strand of a *Hin*dIII (–292) *Bam*HI (+9) fragment (Fig. 1D) was labeled at the *Hin*dIII site and subjected to DNase I footprinting in the presence of Pho4 and/or Pho2 as indicated at the top. Protected regions are indicated on the side.

reported (23). Pho2 binds to its site with similar affinity as to the Pho2 sites adjacent to UASp2. When the two proteins were added together, however, Pho4 binding was significantly increased by the presence of Pho2.

Interestingly, the Pho4 and Pho2 binding sites centered at –185 overlap with a 19 bp palindromic consensus sequence which was proposed to be a phosphate regulated UAS element (5). Four such elements had been identified in the *PHO5* promoter, including UASp1, UASp2 and the –185 region. Since cooperative binding of Pho4 and Pho2 has now been demonstrated at three of these elements, we examined the fourth element (at –469) for Pho4 and Pho2 binding. A very faint footprint at position –468 to –487 was detected only at very high Pho4 concentrations (not shown). This region contains the sequence TATGTG (position –476 to –481) which has two mismatches to the Pho4 consensus binding site. However, this weak Pho4 footprint lies between two Pho2 binding sites and overlaps them partially (Fig. 9). Therefore, we assayed this region for possible cooperative binding of Pho4 and Pho2. Upon simultaneous addition of Pho4 and Pho2, ternary complexes containing Pho4 and one to three molecules of Pho2 were observed (not shown). Although there was clearly some degree of binding cooperativity between Pho2 and Pho4, very high concentrations of both proteins were required for binding. A more quantitative analysis was difficult because of the presence of multiple Pho2 sites. The locations of all Pho4 and Pho2 binding

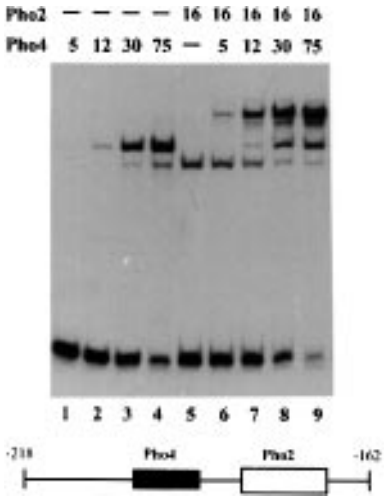


**Figure 8.** Cooperativity between Pho2 and Pho4 is also observed at the newly mapped Pho4 binding site. Binding of Pho4 and Pho2 to an *Ava*II (–218) Acc65 (–162) fragment, containing the Pho4 site mapped in Figure 7 and the adjacent Pho2 site was examined by the gel shift assay as described in Materials and Methods.

sites that were mapped in this work are summarized in Figure 9. Their approximate relative affinity is indicated in the figure.

## DISCUSSION

As outlined in the Introduction, earlier data had suggested that Pho2 might be required for *PHO5* activity, without however actually binding directly to any UAS elements in the *PHO5* promoter. Such a model was based on the strict Pho2 dependence of the *PHO5* promoter, as well as the lack of demonstrable Pho2 binding sites in the *PHO5* promoter, other than a single site which is dispensable for *PHO5* promoter activity. We have now found that there are multiple Pho2 binding sites at the *PHO5* promoter. It is difficult to purify full length Pho2 from *Escherichia coli* extracts, and only after using the HIS-tag technology have we been able to obtain highly purified native Pho2. This may account for our previous difficulties in obtaining clear Pho2 footprints and might explain why Pho2 binding was previously detected only at a region of the *PHO5* promoter where we have now identified a cluster of strong binding sites. Importantly, in the present study we have demonstrated that there is mutual binding cooperativity between Pho2 and Pho4 at each Pho4 binding site, which may resolve the contradiction regarding the Pho2 dependence of the *PHO5* promoter.

A recent paper by Hirst *et al.* (18) demonstrated that a Pho2–VP16 fusion could activate the *PHO5* promoter when it was coexpressed with a Pho4 derivative lacking an activation domain. However from these results, it was not clear whether the Pho2–VP16 fusion was directly contacting DNA or rather was being targeted to the *PHO5* promoter through an interaction with Pho4. Our data indicate that DNA binding of Pho2 is indeed required for its recruitment to the promoter, and furthermore, that Pho2 binding directly reinforces the affinity of Pho4 for each of its binding sites.

Of the different mechanisms invoked to explain the role of Pho2 in the activation of other promoters the one proposed by D. Stillman *et al.* in their studies of the *HO* promoter, where they demonstrated cooperative DNA binding of Pho2 with the zinc finger DNA binding protein Swi5 (12), appears the most relevant for the *PHO5* promoter. In addition, they have shown that the two proteins are important for activity of the *HO* promoter *in vivo*. In contrast, attempts to demonstrate cooperative DNA binding of Pho2 and Bas1 at the *HIS4* promoter *in vitro* were unsuccessful (13). In the *TRP4* promoter, Pho2 has been shown to bind to a site that overlaps a Gcn4 binding site, yet the two proteins appear to bind in a mutually exclusive manner (14).

By sequence comparisons of the Pho2 protected regions at the *PHO5* promoter, as well as those at the *HO*, *HIS4* and *TRP4*

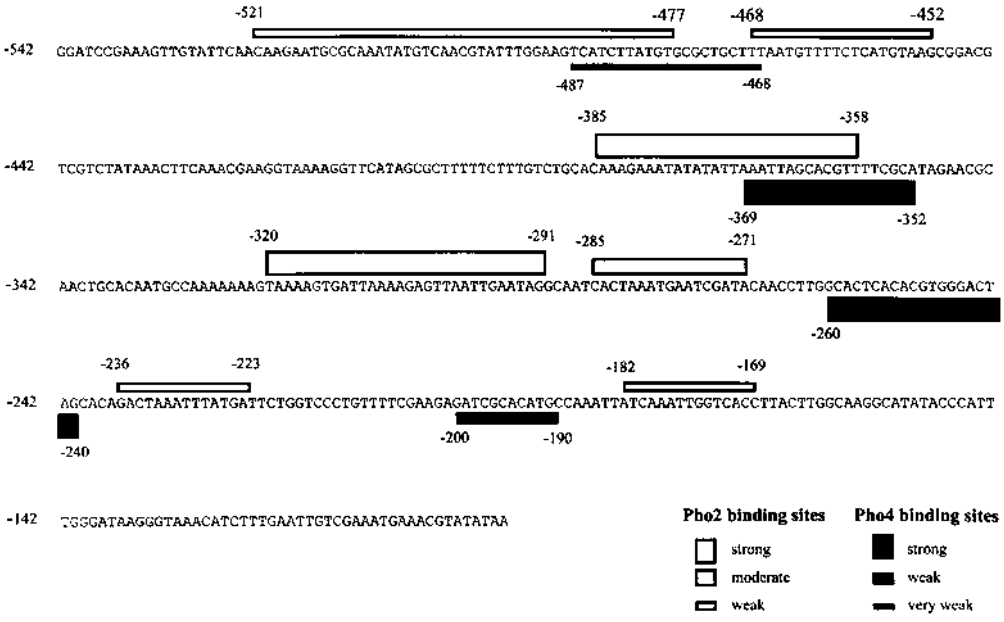


**Figure 9.** Map of Pho4 and Pho2 binding sites at the *PHO5* promoter. The locations of the binding sites as determined in this study are indicated. The height of the bars denotes the relative affinity of the binding sites as determined by the gel shift experiments.

promoters, the following consensus sequence for Pho2 binding emerges: 5′-(T/C)TAA(T/A)T(T/G)AAT-3′. At the *PHO5* promoter, the Pho2 protected region that overlaps with UASp1 and the two regions of protection located between UASp1 and UASp2 contain sequences which fully match the proposed consensus, while the Pho2 sites adjacent to the 3′ side of UASp2 and at the 3′ side of the newly mapped Pho4 site show 1 and 2 mismatches, respectively. The apparent relative affinity of Pho2 for binding sites at the *PHO5* promoter, estimated from gel shift experiments, correlates well with the extent their sequences match the proposed consensus.

A common motif found in the binding sites for many homeodomain proteins is the TAAT sequence (24). Nonetheless, it is clear that sequences outside this core contribute to the binding specificity of particular homeodomain proteins (25–27). The OCT homeodomain proteins, as well as some other homeotic proteins, bind specifically to the sequence TNATTTGCAT (25,28), while the *Drosophila* homeodomain proteins *eve*, *zen*, *en*, *prd* and *ftz* all bind to the consensus sequence TCAATTAAAT (26,29). Both consensus sequences are similar (with two mismatches) to the one proposed here for Pho2 binding. The similar binding specificity of Pho2 and the *Drosophila* homeodomain proteins is in agreement with the high degree of homology in the third recognition helix of Pho2, *eve* and *prd* (30).

## Multiple layers of binding cooperativity

Many homeodomain proteins are promiscuous in their capacity to bind DNA cooperatively with either homologous or heterologous homeodomain proteins and/or with non-homeodomain proteins. Cooperative binding of Pho2 with the zinc finger protein Swi5 was previously demonstrated (12), and here we are reporting cooperative binding of Pho2 with the bHLH protein Pho4, showing that the Pho2 protein can make cooperative interactions with different classes of non-homeodomain proteins. One surprising aspect of the Pho4–Pho2 binding cooperativity is that it appears to be relatively insensitive to the spacing between the Pho4 and Pho2 binding sites. Pho2 facilitated DNA binding by Pho4 regardless of whether the sites overlapped to a significant extent (UASp1), or whether they were separated by up to 12 bp (UASp2). In the *HO* promoter, increasing the space between the Pho2 and the Swi5 binding sites by 10 bp did not influence cooperative binding of these two proteins *in vitro*, nor promoter activity *in vivo* (12). This was interpreted to reflect substantial flexibility of the interaction domains of Pho2 and Swi5, and the same may well apply to Pho2 and Pho4.

The alternative explanation that Pho2–Pho4 interactions alone are sufficient for the formation of a stable ternary complex is ruled out by the experiment showing that binding sites for both proteins are required to observe ternary complexes and cooperative binding (Fig. 6). Interactions between Pho2 and Pho4 have been demonstrated *in vivo* by the two hybrid assay (18), suggesting that Pho2 and Pho4 might bind each other even in the absence of DNA-binding by both proteins. However, the yeast two hybrid system is a very sensitive assay for protein interaction, which can even detect an interaction between a kinase and its substrate (31). Therefore, the yeast two hybrid data probably reflect an interaction that normally occurs when both proteins are bound to DNA.

Pho4 by itself has been reported to bind with lower affinity to a UASp1 oligonucleotide as compared with one containing UASp2 (32). However, as shown here, when larger promoter fragments containing UASp1 or UASp2 were used instead, Pho4 binds to both sites with similar affinity. In comparison, binding to a fragment containing the newly mapped Pho4 site is about three to four times weaker. Except for the core consensus sequence, this site does not show any homology to either UASp1 or UASp2. Deleting (5) or mutating this site (unpublished) does not lead to a significant drop in promoter activity in an otherwise wild-type promoter. This is clearly different from the situation encountered with UASp1 and UASp2. Mutating either of the two elements leads to a 90% reduction in promoter strength (unpublished). In view of the strong cooperative binding of Pho2 and Pho4 to the newly mapped Pho4 site it is still conceivable though that this element is functional *in vivo*, and we are currently testing this possibility.

A role of Pho2 in increasing the affinity of Pho4 for its target sites is also consistent with previous *in vivo* results. Disruption of *PHO2* renders chromatin at the *PHO5* promoter permanently closed, even under phosphate starvation conditions (17). Furthermore, binding of Pho4 to the *PHO5* promoter as assayed by DMS *in vivo* footprinting is lost in a *pho2* strain (9). Overexpression of Pho4, however, restores its ability to bind to its sites and disrupt nucleosomal structure in the *PHO5* promoter even in the absence of Pho2, indicating binding of Pho4 to the UAS elements. Nonetheless, expression of the *PHO5* gene is only ~25% of wild-type level (17). This suggests a role of Pho2 not only in helping Pho4 bind to its target sites but also in enhancing transcriptional activation. A recent report has proposed that interaction between Pho2 and Pho4 might increase the accessibility of the activation domain of Pho4, which might account for this second role (33).

Homeodomain proteins often bind to multiple sites at their target promoters. Five binding sites for the *bcd* protein were found upstream of the hunchback gene (34). Similarly, the Ubx protein binds cooperatively to clusters of its binding sites found in several promoters (35). In many such cases, the binding of the homeodomain protein to multiple sites has been shown to be cooperative. Our binding assays revealed that Pho2 binds cooperatively by itself to a *PHO5* promoter fragment containing two or more Pho2 binding sites, even when those Pho2 binding sites were separated by as much as 50 bp. Cooperative binding to distal sites by other homeobox proteins has been postulated to occur through a DNA looping mechanism (35).

## Role of Pho2 at the *PHO5* promoter in a chromatin context

To fully understand the activation process at the *PHO5* promoter, the chromatin structure of the promoter has to be taken into account. The repressed *PHO5* promoter is covered with four positioned nucleosomes, which are disrupted upon promoter activation in a Pho4-dependent process (36). Although binding of Pho4 to both UASp1 and UASp2 is required for chromatin transition and transcriptional activation to occur (7), it is reasonable to assume that the activation process at the *PHO5* promoter is initiated through UASp1, since it is located in a short nucleosome-free region in the repressed promoter and therefore is available for protein binding under repressing conditions (7). In contrast, UASp2 is located in the middle of nucleosome –2, which in the repressed state prevents binding of Pho4 to this site (7). On the basis of the finding that homeodomains make contacts in the major as well as the minor groove of DNA, it was suggested that

nucleosomes could exclude homeodomain proteins from binding (37). Therefore, only the site overlapping UASp1 is accessible to Pho2 in the repressed *PHO5* promoter and might therefore be of particular importance in *PHO5* regulation. Cooperative binding of Pho2 and Pho4, together with cooperative binding of Pho2 by itself, could make the promoter exquisitely sensitive to small changes in the concentrations of the two regulatory proteins.

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