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Homeodomain–DNA interactions of the Pho2 protein are promoter-dependent

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ABSTRACT

The homeodomain (HD) is a conserved sequence-specific DNA-binding motif found in many eukaryotic transcriptional regulatory proteins. Despite the wealth of *in vitro* data on the mechanism HD proteins use to bind DNA, comparatively little is known about the roles of individual residues in these domains *in vivo*. The *Saccharomyces cerevisiae* Pho2 protein contains a HD that shares significant sequence identity with the *Drosophila* Engrailed protein. We have used the co-crystal structure of Engrailed as a model to predict how Pho2 might contact DNA and have examined how individual residues of the Pho2 HD contribute to transcriptional activation *in vivo* and to DNA binding *in vitro*. Our results demonstrate that Pho2 and Engrailed share many similar DNA-binding characteristics. However, our results also show that some highly conserved residues, which contact the DNA in many HD structures, make relatively small contributions to the DNA-binding affinity and *in vivo* activity of the Pho2 protein. We also show that the N-terminal arm of the Pho2 HD is a critical component in determining the DNA-binding specificity of the protein and that the requirements for residues in the N-terminal arm are promoter-dependent for Pho2 transcriptional activation and DNA binding.

INTRODUCTION

The homeodomain (HD) is a conserved DNA-binding domain found in a large number of eukaryotic transcriptional regulatory proteins and has been identified in organisms ranging from yeast to *Drosophila*, mice and humans (1). Many HD proteins have been extensively characterized *in vitro*, and these studies have provided excellent models for how HDs contact DNA and how mutations in the HD affect the DNA-binding affinity of the protein (2). However, we know comparatively little about how these specific substitutions affect HD function *in vivo*. It is apparent that some HD proteins interact with other transcription factors to bind cooperatively to their *in vivo* target sites (3–7). Depending on the cell type or growth conditions, some of these proteins interact with one of several cofactors and different combinations of these proteins function to regulate different sets of genes. These protein interactions may influence the target site specificity or mechanism of DNA binding by the HD protein such that the activity of these proteins is sometimes different *in vivo* from that observed *in vitro*. It is therefore important to investigate the roles of individual residues in the HD *in vivo*, as well as *in vitro*, to understand the mechanisms of HD function.

Pho2, also known as Bas2 and Grf10, is a HD protein in the yeast *Saccharomyces cerevisiae* that is involved in the transcriptional regulation of a variety of metabolic pathways, including adenine, histidine, tryptophan, sulfate and phosphate metabolism (8–12). Pho2 is also required for proper expression of *HO*, the gene encoding the restriction endonuclease that initiates cell type switching in yeast (6,13). Pho2 specifically binds to sites in the promoters of the genes it regulates and acts as a co-activator with other transcription factors, such as Pho4, Bas1 and Swi5 (6,9,11,13–17). Pho2 also functions independently as an antagonist of Gcn4 (10). Pho2 is similar in many respects to HD proteins from higher eukaryotes which interact with several different cofactors to possibly regulate different sets of genes. The analysis of Pho2, therefore, provides a model system to examine the contribution of individual residues in a HD to the expression from several different promoters *in vivo*, as well as HD protein–DNA interactions *in vitro*.

The Pho2 protein contains a HD that shares 37% identity and 62% similarity with the *Drosophila* Engrailed HD (Fig. 1). Residues that contact DNA in the Engrailed co-crystal structure are either identical or highly conserved to those in the Pho2 HD (18,19). The yeast α2 and Engrailed HDs show an even lower degree of sequence similarity than between the Pho2 and Engrailed HDs. However, despite this low sequence similarity, structural studies have shown that the Engrailed and α2 HDs adopt similar structural folds and make many identical contacts to DNA (20). Therefore, it seems reasonable that Pho2 adopts a conformation similar to the Engrailed HD and that DNA contacts made by the Engrailed HD could serve as a model for Pho2–DNA contacts.

In this paper, we have constructed a series of amino acid substitutions in the Pho2 HD to investigate the mechanism that Pho2 uses to bind DNA. The effects of these substitutions were examined on Pho2-dependent transcriptional activation of different promoter

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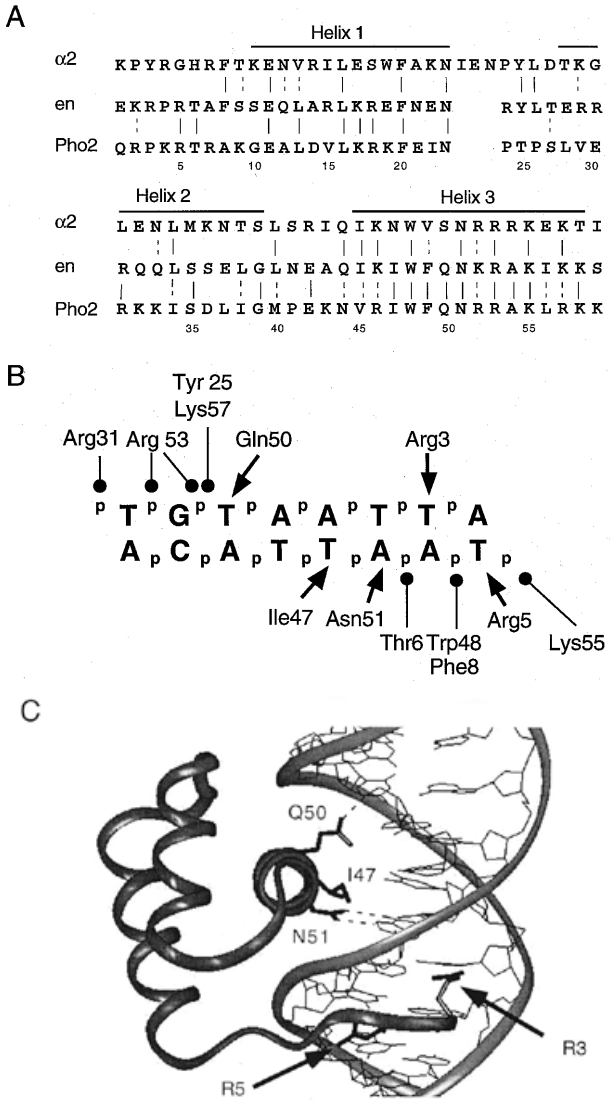


Figure 1. Sequence alignment of the Pho2, α2 and Engrailed HDs and DNA contacts of the Engrailed HD. (**A**) The sequence alignment of the α2, Engrailed and Pho2 HDs is shown. Identical amino acids are indicated by solid lines; conserved amino acids are indicated by dashed lines. Helical regions observed in the Engrailed and α2 co-crystal structures are indicated (19,20). (**B**) Illustration of DNA contacts in the Engrailed co-crystal structure (19). Arrows represent base-specific contacts and circles represent sugar–phosphate backbone contacts. (**C**) A model of the Engrailed HD binding to DNA derived from the co-crystal structure (19). Side chains that make base-specific contacts in the major groove (Q50, N51, I47) and minor groove (R3 and R5) have been displayed.

elements *in vivo* and DNA-binding affinity *in vitro*. In general, our results support the predictions based on the Engrailed co-crystal structure and agree very well with mutational analysis of the Engrailed HD (19,21,22). Many highly conserved residues in the HD of Pho2 are essential for Pho2-dependent activation at the different promoters. However, our results also suggest that contributions of other highly conserved residues, which contact DNA in other HD proteins, are relatively small *in vivo*. In addition, we observe that substitution of residues in the N-terminal arm of the HD have differential effects on the various Pho2-dependent promoters *in vivo* and DNA-binding affinity *in vitro*. This result suggests that the mechanism of DNA binding by Pho2 is in part determined by its target site.

MATERIALS AND METHODS

Yeast strains

The isogenic yeast strains L4196 (*MAT**a** leu2-2 ura3-52*), L4198 (*MATα pho2 leu2-2 ura3-52*) and L4224 (*MATα pho2 gcn4 leu2-2 ura3-52*) were generously provided by G.Fink (11). Yeast strains were transformed by standard procedures (23) and low-phosphate media were prepared as described (24).

Plasmids

Mutations in *PHO2* were constructed and expressed in yeast using plasmid pMCJ8, a *CEN4*, *LEU2* plasmid containing a modified version of the 2.3 kb *Mlu*I–*Cla*I fragment of *PHO2*. This construct carries a *PHO2* DNA fragment from 386 bp upstream of the ATG, which includes the entire *PHO2* promoter, to 235 bp downstream of the *PHO2* termination codon. The coding region of *PHO2* in pMCJ8 has been modified so that unique restriction sites exist every 30–50 bp in a 600 bp region (*Nsi*I–*Nco*I). This construct also contains the coding sequence for the HA epitope inserted into the *Bsi*WI site of *PHO2*. To construct pMCJ8, a 2.3 kb *PHO2* fragment was amplified by PCR with primers that contain *Pvu*II sites and anneal adjacent to the *Mlu*I (5′ primer) and *Cla*I (3′ primer) sites. The PCR product was subcloned into the *Pvu*II site of a derivative of YCplac111 (25) that lacks the polylinker region and in which the *Bgl*II site had been destroyed by filling in with Klenow polymerase (pMCJ1). To insure that the clone did not contain any PCR derived mutations, a genomic *Mlu*I–*Cla*I fragment was used to replace PCR generated DNA to construct plasmid pMCJ6. This *PHO2* construct was then modified by recursive PCR with 10 overlapping oligonucleotides which create multiple unique restriction sites throughout the region coding for the Pho2 HD (26). The restriction sites were designed such that only silent codon changes were introduced into the gene. This reaction was followed by PCR amplification with external primers to generate the specific full length gene. This product was then digested with *Nsi*I and *Nco*I and inserted into the backbone of pMCJ6. Regions containing sequence errors were repaired by cloning of double stranded oligonucleotides containing the correct sequence, and the entire clone was verified by sequence analysis. The final cloning step was the insertion of a double stranded oligonucleotide encoding the HA epitope into the *Bsi*WI site of *PHO2* to generate a single HA tag at the N-terminus of Pho2. This construct was verified by DNA sequencing, western analysis and the ability to activate *PHO5* expression in comparison to wild-type *PHO2*.

Transcriptional activation by the *PHO5*, *HIS4* and *HO* Pho2-dependent UAS elements (6,9,13,27,28) were assayed by cloning these sites into pTBA30, a derivative of a *CYC1-lacZ* promoter fusion vector lacking its UAS elements (29). Complementary oligonucleotides corresponding to the Pho2-dependent UAS elements, including TCGA overhangs at their 5′-ends (Table 1), were synthesized, annealed and cloned into the *Xho*I site of pTBA30 according to established methods (30). The orientation and number of UAS elements was verified by DNA sequencing. The *ADE1-lacZ* reporter vector used in this study, p115, was provided by G.Fink (11).

Table 1. UAS elements used in UAS-*lacZ* reporters and EMSAs

Site[a]	Sequence[b]
	Pho2 Pho4
UASp1	tcgaTATTAAATTAGCACGTTTTCGCATAGA
	Pho2 Swi5
UAS_{HO}	tcgaCATCAATTTAAAAAAAAAACCAGCATGCTATAATGCTGGAGCAAAG
	Bas1 Pho2 Rap1
UAS_{HIS4}	tcgaAACTGACTCTAATAGTGACTCCGGTAAATTAGTTAATTAATTGCTAAACCCATGCACAGTGACTCACGTTTT

[a]Pho2 and cofactor binding sites were defined in refs 7,8,14,41,53.
[b]Sequence of the top strand of oligonucleotides used in constructing UAS-*lacZ* reporters and in EMSAs for each site is shown. Lower case letters indicate bases added for cloning the sites into reporter vectors.

Plasmid pSEA100/QK50, which was used for expression in bacteria of the Engrailed HD altered-specificity mutant QK50, has been described (21). Plasmid pMCJ83 contains the K55A mutation in pSEA100/QK50 and was constructed by insertion of a double stranded oligonucleotide with the appropriate codon change into the *Bss*HII–*Cla*I sites of pSEA100/QK50.

Plasmid pMCJ47 was used for yeast expression of a Pho2–Engrailed hybrid protein and was constructed by replacing the Pho2 HD (residues 77–135) in pMCJ8 with the Engrailed HD. Primers were designed containing sequences specific to both *PHO2* and Engrailed in pSEA100 (21) so that the appropriate spacing of the Engrailed HD coding region within *PHO2* was maintained. The resulting PCR product was cloned into the *Nsi*I and *Bam*HI sites of pMCJ8. The Pho2–Engrailed A7R mutant was constructed by insertion of double stranded oligonucleotides containing the appropriate codon change as described above. Clones were verified by DNA sequencing and western analysis. Plasmid pMCJ45 was used for yeast expression of a Pho2–α2 hybrid protein and was generated in a manner similar to the Pho2–Engrailed construct, except that sequence encoding the Pho2 HD (residues 77–134) was replaced with PCR derived DNA encoding the α2 HD generated from plasmid pAV115 (31). The Pho2–α2 R7A mutant was constructed by the same manner using the α2 R7A mutant as a template (31).

Acid phosphatase and β-galactosidase assays

Acid phosphatase assays (APase) were performed on cells grown to mid log phase as previously described (24). Three independent transformants were assayed in each experiment. Whole cell extracts were assayed for APase activity with *p*-nitrophenyl phosphate as the substrate. One unit of activity was defined as the amount producing 1 μmol of *p*-nitrophenol per hour per absorbance unit at A_{600} of cells. β-Galactosidase activity was measured as described (32). Chloroform and SDS-permeabilized whole cell extracts were assayed for *lacZ* expression with *O*-nitrophenyl-β-D-galactosidase as the substrate. Units were calculated using the A_{600} value of the culture for normalization. The values presented in the tables are the percent activity for each mutant in comparison with wild-type Pho2 and were calculated using the formula % = 100 × ($Units_{HDmutant}$ − $Units_{\Delta Pho2}$)/($Units_{WT}$ − $Units_{\Delta Pho2}$). Assays of the *PHO5* and UASp1 reporters were performed in strain L4198 under low phosphate conditions. The UAS_{HIS4} and *ADE1* reporters were assayed in strain L4224 in SD media without adenine, uracil and leucine. The UAS_{HO} reporter was assayed in strain L4198 in SD media without uracil and leucine.

Protein expression and purification

The plasmid pGrf10:His was used for expression of recombinant His-tagged Pho2 (6). Plasmids for bacterial expression of recombinant His-tagged Pho2 HD mutants were constructed by replacing the *Nsi*I–*Nco*I region of pGrf10:His with *Nsi*I–*Nco*I fragments from selected HD mutants.

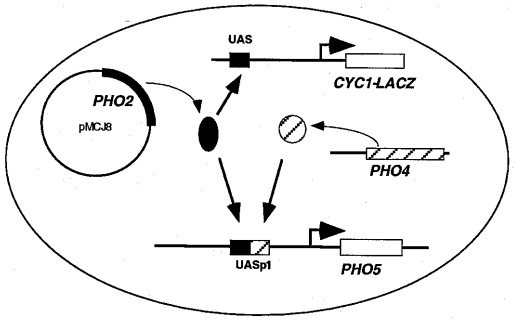
Purification of recombinant His-tagged Pho2 and all HD mutants was performed as described (6) with the following modifications. BL21-pLysS *Eschericia coli* cells harboring the appropriate expression vector were diluted 1:100 into 250 ml of Luria broth. Cells were induced for 4 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside, harvested and resuspended in 10 ml of HIS binding buffer (20 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.1% Nonidet and 1 mM PMSF). The filtered extract was loaded on a 0.1 ml nickel–sepharose (Qiagen) column equilibrated in HIS binding buffer. After loading, the column was washed extensively with HIS binding buffer. The column was then washed with 10 vol of HIS binding buffer containing 60 mM imidazole, followed by elution in 1 ml buffer containing 1 M imidazole. The eluant was desalted with a Sephadex G-25 NAP 10 column (Pharmacia) equilibrated with AN100 buffer (20 mM Tris pH 8.0, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 100 mM NaCl and 1 mM PMSF). Proteins were estimated at 80–90% purity by Coomassie stained SDS–PAGE and quantitated using the BioRad protein assay reagent with BSA as a standard.

The recombinant wild-type Engrailed and Engrailed K55A mutant proteins were purified from *E.coli* as described (21). Fractions were assayed by EMSAs and SDS–PAGE and the final purity of the proteins were estimated at ~90%.

Western blots

Whole yeast cell extracts were prepared from 25 ml cultures of cells grown in SD media lacking leucine. Following centrifugation, cells were washed in ice-cold lysis buffer [100 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM 2-mercaptoethanol, 10% glycerol (w/v), 5 mM $MgCl_2$, 1 mM PMSF, 10 mg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, 0.3 mg/ml benzamidine, 2 mg/ml pepstatin A] at 2 ml buffer per gram of cells. Cells were resuspended in 0.5 ml lysis buffer, disrupted with glass beads by vortexing six times for 30 s, and returned to ice. Extracts were recovered from the beads and clarified by centrifugation at 15 000 *g* for 30 min at 4°C. Protein concentrations were determined with BioRad protein assay reagent using BSA as a standard and the lysates were adjusted to the same concentration. Protein from each lysate was electrophoresed on an 8% SDS–PAGE gel, electroblotted

A



B

Cofactor	Pho4		Bas1		Swi5
Reporter	*PHO5*	UASp1-*lacZ*	UAS_{HIS4}-*lacZ*	*ADE1-lacZ*	UAS_{HO}-*lacZ*
Assay	APase	β-Gal.	β-Gal.	β-Gal.	β-Gal.
pMCJ1 (-*PHO2*)	0.2 U	0.4 U	12 U	5 U	3.0 U
pMCJ8 (+*PHO2*)	3.6 U	65 U	655 U	42 U	7.8 U
Fold Activation	15 X	161 X	52 X	8 X	2.6 X

Figure 2. *In vivo* assays of Pho2 activity. (**A**) Schematic representation of the *in vivo* assay used for measuring the activity of Pho2 HD mutants. pMCJ8, which contains the cassetted *PHO2* gene, and one of the Pho2-dependent *CYC1-lacZ* reporter constructs were co-transformed into *pho2* deletion strains. Expression of *lacZ* was measured from Pho2-dependent *CYC1-lacZ* reporters and acid phosphatase activity from the endogenous *PHO5* gene. (**B**) Results from APase and β-galactosidase assays measuring the effects of pMCJ8 (*PHO2*) and pMCJ1 (no *PHO2*) on *PHO5* and UASp1-*lacZ* expression under low phosphate conditions; UAS_{HIS4}-*lacZ* and *ADE1-lacZ* under amino acid starvation conditions; and UAS_{HO}-*lacZ* during vegetative growth. Assays were performed as described in Materials and Methods and values shown are the units of APase or β-galactosidase activity that are the average of three independent assays. The standard deviation is <20%. The fold activation was calculated by comparing the units of activity in the presence and absence of *PHO2* (*PHO2/pho2*).

to nitrocellulose filters and the filters were blocked with 5% dry milk. Monoclonal antiserum specific to the HA epitope, 12CA5-H (Babco Industries), was used as a primary antibody, with goat anti-mouse antiserum conjugated to horse radish peroxidase as the secondary antibody. Westerns were visualized by chemiluminesence (Amersham ECL) and exposed to film.

Electrophoretic mobility shift assays

EMSAs were performed with complementary oligonucleotides that were used in constructing *CYC1-lacZ* reporter plasmids. The recessed 3′-ends of the annealed oligonucleotides were filled with dATP, dGTP, dTTP and [^{32}P]dCTP by Klenow polymerase. Labeled oligonucleotides were purified over Sephadex G-25 spin columns (Boehringer Mannheim). Approximately 1 ng of labeled double-stranded oligonucleotide was added to 50 µl reactions containing 100 mM NaCl, 25 mM Tris–HCl pH 7.5, 4% (w/v) glycerol, 2 mM 2-mercaptoethanol, 0.05% Triton X-100, 0.1 mM EDTA, 1 mM PMSF and 1 µg of non-specific competitor, poly (dIdC)·poly (dIdC) (Pharmacia). Serial dilutions (5-fold) of recombinant protein diluted in 1 mg/ml BSA, 20 mM Tris pH 8.0, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 100 mM NaCl and 1 mM PMSF, were added to the label mix and the reactions were incubated for 30 min at room temperature. The reactions were then loaded onto 6% polyacrylamide gels in 0.5× TBE and the gels were electrophoresed at 160 V for 2 h at room temperature. The dried gels were exposed to a phosphor screen, and visualized on a Molecular Dynamics Phosphorimager. The relationship of the signal and intensity of the images shown in Figures 4 and 5 are linear over a range between 10 and 5000.

RESULTS

In vivo assay for Pho2

To examine the contributions of individual residues within the Pho2 HD, we developed an assay system that allows us to measure Pho2 activity at its different target sites *in vivo* (Fig. 2A). A wild-type *PHO2* gene that contains unique restriction sites throughout the HD coding region was constructed so that specific amino acid substitutions could be introduced using double-stranded oligonucleotides. The engineered *PHO2* construct fully complements a *pho2* null mutation and has phosphate regulated Pho5 APase activity equivalent to the wild-type *PHO2* strain (data not shown). These results indicate that codon changes in the engineered *PHO2* construct or expression of *PHO2* from a plasmid do not noticeably affect the activity of Pho2 protein in the cell.

We wished to examine the effects of substitutions in Pho2 on activation of the endogenous *PHO5* gene as well as at several other Pho2-dependent promoters. To this end, we have constructed transcription reporter vectors containing Pho2-dependent UAS elements from the *PHO5, HIS4* and *HO* promoters which activate expression of *lacZ* in the context of a heterologous promoter and have obtained an *ADE1-lacZ* promoter fusion (6,9,11,15,33). To establish that the UAS-*lacZ* constructs are dependent on Pho2, we measured the ability of the engineered Pho2 to activate transcription of the various Pho2-dependent UAS elements and the endogenous *PHO5* gene (Fig. 2B). Expression from each of the *lacZ* reporter constructs and *PHO5* was activated in the presence of Pho2 (pMCJ8) when compared with cells assayed under the same conditions in the absence of Pho2 (pMCJ1). These results agree with published data on Pho2-dependent activation (6,9,11,15,33) and demonstrate that this assay system can be used to measure the *in vivo* effects of mutations in the Pho2 HD.

Conserved residues in the Pho2 HD are essential for function of Pho2-dependent promoters

To examine the contribution of individual residues in the HD to Pho2 activity *in vivo*, we have constructed a series of alanine substitutions at positions that would be predicted to contact DNA by comparison with the Engrailed HD co-crystal structure (19). These mutants were assayed for their effects on transcriptional activation of several Pho2-dependent reporters (Table 2). In general, most of the results agree with the predictions based on the co-crystal structure and mutational analysis of the Engrailed HD (19,21). For example, with the exception of the *ADE1-lacZ* reporter, alanine substitutions of the highly conserved residues W48, N51 and R53 result in almost a complete loss of expression of *PHO5* and the Pho2-dependent *lacZ* reporters (Table 2). Western blot analysis of yeast lysates showed that the Pho2 mutants are expressed at approximately wild-type levels, indicating that the substitutions do not alter the level of the proteins in the cell (Fig. 3). These results support the observation that Pho2 contains a prototypical HD since these residues are found in over 95% of all HDs and are considered to be virtually invariant.

Table 2. Promoter activation by Pho2 mutants

Cofactor		Pho4		Bas1		Swi5
Reporter		*PHO5*	UASp1	UAS_{HIS4}	*ADE1-lacZ*	UAS_{HO}
Assay[a]	Contact[b]	APase	β-gal	β-gal	β-gal	β-gal
WT		100	100	100	100	100
R2A		65	34	117		
K4A		51	16	57		
R5A	T7	7.0	0.5	41	83	0.0
T6A	P	67	34	74	135	75
R7A		10	7.4	69		
T25A	P	76	63	64		
T25Y	P	122	321	112		
R31A	P	20	3.9	49		
I47A	T4	18	0.3	17	51	4.2
W48A	P	1.0	0.2	2.1	31	0.0
Q50A	T2	33	4.7	45	43	33
N51A	A5	1.3	0.0	2.8	34	0.0
R53A	P	2.7	0.2	5.0	42	0.0
K55A	P	79	43	68	111	83
R57A	P	37	7.9	58	120	41
K58A		57	20	60		
K59A		95	114	81		

[a]Values shown for each mutant are the percent activity of wild-type Pho2 and were calculated as described in Materials and Methods.
[b]Contacts of each residue are predicted from the Engrailed co-crystal structure (19). P represents a phosphate-backbone contact; A, a base-specific contact to adenine; T, a base-specific contact to thymine.

Although alanine substitutions of W48, N51 and R53 result in almost a complete loss of transcriptional activation of the *PHO5*, UASp1 and UAS_{HO} Pho2-dependent reporters, there is still significant Pho2-dependent expression at the Bas1/Pho2-dependent *ADE1-lacZ* reporter. Previous studies (11), along with the results shown in Figure 2, demonstrate that expression of *ADE1* is Pho2-dependent. However, Pho2 has never been shown to bind the *ADE1* promoter in EMSAs or in DNase I protection experiments. In addition, it has recently been shown that a *pho2* mutant, which completely lacks the HD, is able to work in combination with the Bas1 protein to partially activate expression of the *ADE5* promoter (34). Therefore, it is possible that *ADE1* expression may be less dependent on Pho2 HD–DNA interactions than the other genes regulated by Pho2 and that other regions of the protein may be sufficient for the residual activation in combination with Bas1.

In the Engrailed co-crystal structure, residue Q50 makes a base-specific contact to a thymine and has been shown to be an important determinant of DNA-binding specificity in the Engrailed HD, as well as in other HDs (19,21,35–37). However, we observe that alanine substitution of this residue in Pho2 results in an ~2–3-fold decrease in the level of expression of the *PHO5*, UAS_{HIS4}, *ADE1-lacZ* and UAS_{HO} reporters. This result is consistent with previous observations that alanine substitution of this residue in the Engrailed HD causes only a 2-fold reduction in the DNA-binding affinity *in vitro*. These results suggest that while this residue is an important determinant in DNA-binding specificity, it does not make a large contribution to the DNA-binding affinity of the protein (21). Although the Pho2 Q50A mutation has only mild effects on the expression of the *PHO5*, UAS_{HIS4}, *ADE1-lacZ* and UAS_{HO} reporters, expression from the UASp1 reporter is ~5% of its wild-type level. The low level of activation by the Q50A mutant at UASp1 might be due to different protein–protein or protein–DNA interactions which take place in the context of the full promoter as compared to the isolated UASp1 element. This is supported by recent observations that Pho2 binds by itself and cooperatively with Pho4 at multiple sites in the *PHO5* promoter (17).

The effects of mutations at other positions in the Pho2 HD vary with some substitutions resulting in intermediate levels of Pho2-dependent expression, while other mutants show nearly wild-type activity even though they are modeled to contact the DNA. For example, in the Engrailed co-crystal structure residues T6 and K55 make contacts to the sugar-phosphate backbone of the DNA. Alanine substitutions of these residues in Pho2 cause <2-fold decrease in the level of expression from most of the Pho2-dependent reporters. This result suggests that if these residues in Pho2 contact the DNA, then they only make relatively small contributions to the overall DNA-binding affinity. One explanation for the effect of these mutations is that in Pho2, residues at other positions contact the DNA and, therefore, replace the function of these positions in Engrailed. In order to test this model, alanine substitutions were made in Pho2 at residues

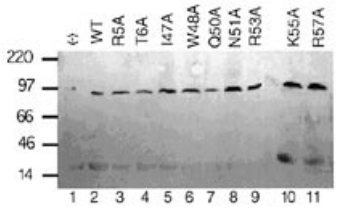


Figure 3. Western blot of Pho2 HD mutants. Yeast cell extracts containing the Pho2 mutants were normalized for protein concentration, electrophoresed on an 8% SDS–PAGE and blotted to nitrocellulose. The filter was then probed with antisera to the HA epitope and cross-reacting proteins was visualized by chemiluminesence. Lane 1 contains protein from an extract prepared from cells harboring pMCJ1 (-*PHO2*). Lane 2 contains protein from an extract prepared from cells harboring a plasmid containing the wild-type engineered *PHO2* (pMCJ8). Lanes 3–12 contain extracts from cells harboring the R5A, T6A, I47A, W48A, Q50A, N51A, R53A, K55A and R57A Pho2 HD mutants.

which are in close proximity to the DNA in the Engrailed co-crystal structure, but do not make a direct contact to the DNA. For example, in the N-terminal arm of Pho2, it is possible that residues R2 and K4 could contact the DNA and, therefore, replace the function of T6 in Engrailed. Likewise, residues K58 and K59 in helix III of Pho2 could substitute for the function of K55 in the Engrailed HD. Alanine substitutions of Pho2 residues R2, K4 and K58 result in a moderate loss of expression of both the *PHO5* and UASp1 reporters, which shows that these residues make a contribution to Pho2 HD–DNA interactions (Table 2). On the other hand, the K59A mutant activates transcription to nearly wild-type expression levels, suggesting that it is not required for Pho2 function.

In the structures of the *Drosophila* Engrailed, Antennapedia, Even-skipped, Paired and yeast α2 HDs, residue 25 is a tyrosine located in the turn between helix I and helix II that extends from the HD and makes a contact to the phosphate backbone (19,20,38–40). In Pho2, residue 25 is a threonine and although it is capable of making a hydrogen bond with the backbone, its length is significantly shorter and, therefore, it may not be capable of making an optimal contact to the DNA. A T25A substitution in Pho2 causes a moderate decrease in the level of expression of the reporters tested, which indicates that residue T25 makes a weak contribution to the *in vivo* activity of Pho2 (Table 2). On the other hand, a tyrosine substitution at this position, T25Y, results in a noticeable increase in the level of expression of the reporters and in particular at the UASp1 where expression increases 3-fold over the wild-type level (Table 2). This result confirms the predictions from the Engrailed crystal structure, and suggests that while Pho2 residue T25 is making a contact with the DNA, it may not be optimal.

Substitutions in the HD N-terminal arm have differential effects on the expression of the Pho2-dependent reporters

Residue R5 is conserved in >95% of all HD proteins and makes a hydrogen bond to a thymine in the minor groove of the DNA in the Engrailed co-crystal structure (1,2,19). As expected, alanine substitution of R5 results in almost a complete loss of activation from *PHO5*, UASp1 and UAS_{HO} promoter elements (Table 2). In contrast, this same mutant retains ~40–85% of its activity at the Bas1 and Pho2-dependent *ADE1-lacZ* and the UAS_{HIS4} reporters. Likewise, alanine substitution of R7 results in very low levels of

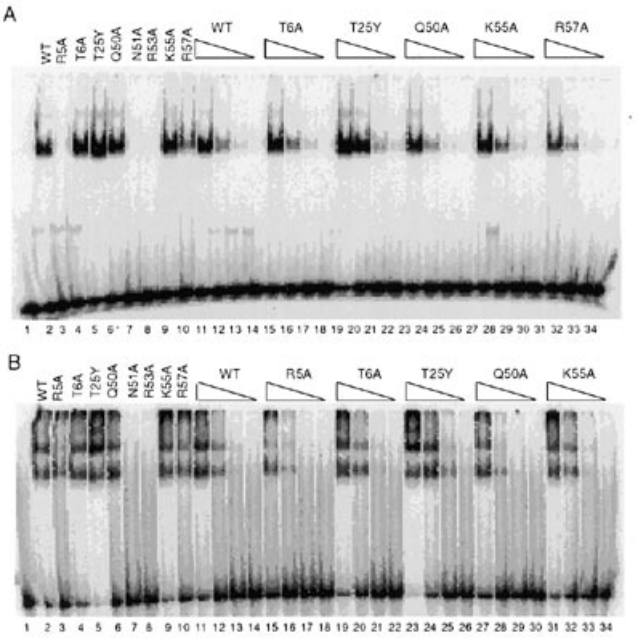


Figure 4. DNA binding of Pho2 mutants to the UASp1 and UAS_{HIS4} binding sites. (**A**) UASp1 binding of Pho2 HD substitution mutants. Lane 1, no protein; Lanes 2–10 contain equal concentrations (1.8×10^{-7} M) of purified wild-type, R5A, T6A, T25Y, Q50A, N51A, R53A, K55A and R57A Pho2 proteins bound to radiolabeled UASp1. Lanes 11–34 contain 5-fold titrations of wild-type, T6A, T25Y, Q50A, K55A and R57A Pho2 proteins ranging from (1.8×10^{-7} M to 1.4×10^{-9} M) bound to UASp1. (**B**) UAS_{HIS4} binding to Pho2 HD mutants. Lanes 2–10 contain equal concentrations (1.8×10^{-7} M) of purified wild-type, R5A, T6A, T25Y, Q50A, N51A, R53A, K55A and R57A Pho2 proteins bound to radiolabeled UAS_{HIS4}. Lanes 11–34 contain 5-fold dilutions of wild-type, R5A, T6A, T25Y, Q50A and K55A Pho2 proteins ranging from (1.8×10^{-7} M to 1.4×10^{-9} M) bound to UAS_{HIS4}.

expression from *PHO5* and the UASp1 reporter, while the UAS_{HIS4} reporter retains 69% of its expression. These results show that the requirements for residues in the N-terminal arm vary at the different promoter elements. Furthermore, it suggests that the function of N-terminal residues is not the same when Pho2 is acting with Bas1 compared to when it is acting with Pho4 or Swi5.

In vitro assays of Pho2 mutants correlate with the observed effects *in vivo*

The analysis described above showed the effects *in vivo* of substitutions in the Pho2 HD. We next wished to determine how these mutations affect Pho2 binding *in vitro*, and how well these results correlate with the *in vitro* analysis of Engrailed HD mutants (21,22). Wild-type Pho2 and selected substitution mutants were assayed for DNA-binding activity using purified protein with radiolabeled Pho2-dependent UAS elements. The results from the EMSAs show a good correlation between the level of Pho2-dependent transcriptional activation *in vivo* and DNA-binding affinity *in vitro* (Fig. 4). Those mutants that have decreased *in vivo* activity also have a corresponding loss in DNA-binding affinity. For example, the proteins containing the mutations R5A, N51A and R53A show an ~100-fold decrease in the DNA-binding affinity for the UASp1 site (Fig. 4A, lanes 3, 7

and 8). These results are consistent with *in vitro* studies on the Engrailed HD which show a similar decrease in DNA-binding affinity with the R5A and N51A substitutions in Engrailed (22). Other substitutions in Pho2, such as T6A and K55A, which retain significant levels of activity *in vivo*, have approximately wild-type DNA-binding affinity for the UASp1 site *in vitro* (Fig. 4A, lanes 4 and 9). Since these substitutions only have a small effect on Pho2 binding, we were interested in whether these same changes would also have a minor effect on DNA-binding affinity of the Engrailed HD. EMSAs with the purified proteins showed that the K55A substitution in the Engrailed HD only reduces the DNA-binding affinity to the Engrailed binding site by 1.4-fold (data not shown). The T6A substitution also binds DNA with almost wild-type affinity (S.Ades and R.T.Sauer, personal communication). Therefore, the effects of T6A and K55A mutants in Pho2 are similar to the effects of the same substitutions in Engrailed, which suggests these residues may be involved in similar contacts in both proteins and that they make a relatively small contribution to the overall DNA-binding affinity.

The T25Y substitution, which functions as a better transcriptional activator than the wild-type protein, binds DNA with 4-fold higher affinity than the wild-type protein (Fig. 4A, lanes 19–22). This result again supports the model that while T25 interacts with the DNA, it does not appear to be an optimal contact.

In general, the apparent DNA-binding affinity of the Pho2 mutants *in vitro* correlates with the observed effects of these mutants *in vivo*. One exception to this correlation is observed with Pho2 Q50A DNA-binding to the UASp1 site. This mutant binds the UASp1 site at 70% of wild-type levels, but is only able to activate expression of the UASp1 reporter *in vivo* at 5% of wild-type levels. The small reduction in DNA-binding affinity of the Pho2 Q50A mutant is similar to the ~2-fold reduction in DNA-binding affinity that was observed by the Q50A substitution in the Engrailed HD (21). Site selection experiments of the Engrailed Q50A mutant show that it has relaxed sequence specificity. If the Pho2 Q50A mutant has similar relaxed specificity, then it may not be able to distinguish its natural binding sites from other binding sites *in vivo*. That may explain why the Q50A mutation has a large effect on activation of the UASp1 reporter. Interestingly, the Q50A mutant only has a minor effect, 3-fold, on activation of *PHO5*. This result suggests there may be other factors that contribute to the specificity of Pho2 DNA binding to the full *PHO5* promoter which are not present in the isolated UASp1 element. A second UAS element, UASp2, has been identified within the *PHO5* promoter that is strongly bound by Pho4 but only weakly by Pho2 (14,33). It is possible that Pho4 binds to this site and that interactions between Pho4 and Pho2 compensate for the loss of DNA-binding specificity by the Pho2 Q50A mutant (14,17). Pho4 does not bind tightly to the UASp1 element, so these protein interactions would not be able to compensate for the effects of the Q50A on the UASp1 reporter.

Since the effects of some of the Pho2 mutants on the *PHO5* and UASp1 reporters are different when compared with the UAS_{HIS4} or *ADE1-lacZ* reporters, we examined the DNA-binding affinity of the mutants for the UAS_{HIS4} site (Fig. 4B). Most of the mutants tested showed approximately the same binding affinity for the UAS_{HIS4} and UASp1 sites. However, there is a significant difference in the DNA-binding affinity of the R5A mutant for the UASp1 and UAS_{HIS4} sites (Fig. 4B, lanes 3 and 15–18). No binding of the R5A mutant is detected to the UASp1 site, while R5A binding to the UAS_{HIS4} site is only reduced ~3-fold when compared with the wild-type protein (Fig. 4B, lanes 11–14 versus 15–18). This result correlates well with the differences observed *in vivo* at the UASp1 and UAS_{HIS4} reporters (Table 2), and further supports the finding that N-terminal residues are not required to the same extent for Pho2 DNA-binding at the *HIS4* and *ADE1* promoters compared to other Pho2-dependent promoters.

The Engrailed HD can substitute for the Pho2 HD

The results described above show that many amino acid substitutions in the Pho2 HD have the effects that were predicted from a model of the Engrailed co-crystal structure. These results suggest that the mechanism and specificity of DNA-binding by these two proteins is very similar. To further test this model, we replaced the Pho2-HD with the Engrailed HD and assayed for the ability of the Pho2–Engrailed chimera to activate expression of endogenous *PHO5*, along with the UASp1 and UAS_{HIS4} reporter constructs (Table 3). Although the Pho2–Engrailed hybrid activates the UAS_{HIS4} reporter construct to 61% of wild-type levels, it fails to activate *PHO5* or the UASp1 reporter construct. This result was not unexpected since Engrailed has an alanine at residue 7 and our results show that the R7A substitution in Pho2 results in almost a complete loss of expression from *PHO5* and UASp1 while the UAS_{HIS4} reporter retains 69% of its activity (Table 2). An Arg at position 7 in the HD may therefore be essential for the Pho2–Engrailed protein to function at the *PHO5* promoter and UASp1 site. To test this model, an A7R substitution in the Pho2–Engrailed hybrid was constructed and assayed *in vivo*. This mutation results in a protein that activates the expression of *PHO5* to 63% of wild-type level and the UASp1 construct to 32% of wild-type level (Table 3). We also observe an increase in the level of UAS_{HIS4} reporter expression with this mutant. These results demonstrate the importance of the HD N-terminal arm in DNA binding, and further show that there are different sequence requirements for residues in the N-terminal arm of the HD at the various Pho2-dependent promoters.

Table 3. Promoter activation by Pho2 hybrid proteins

Cofactor	Pho4		Bas1
Reporter	*PHO5*	UASp1-*lacZ*	UAS_{HIS4}-*lacZ*
Assay[a]	APase	β-gal	β-gal
Wild-type	100	100	100
Pho2–Engrailed	4.9	1.1	58
Pho2–Engrailed A7R	68	27	118
Pho2–α2	20	3.0	7.9
Pho2–α2 R7A	1.4	0.7	2.0

[a]Values shown are percent activity of wild-type Pho2 and were calculated as described in the Materials and Methods.

The results shown above indicate that the Engrailed HD can at least partially replace the function of the Pho2 HD. We next wanted to determine what effect a HD substitution with different DNA-binding specificity would have on the expression of the Pho2-dependent reporters. To address this question, the Pho2 HD was replaced with the HD from the yeast α2 repressor. Although, the Pho2 and α2 HDs share some protein sequence similarity, most of the residues that make base-specific contacts are not conserved

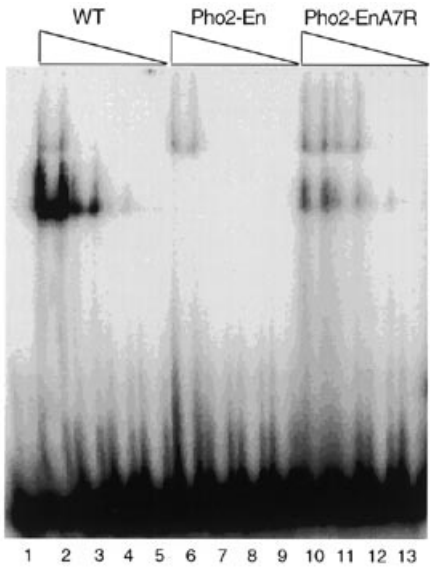


Figure 5. DNA binding of wild-type Pho2, Pho2–Engrailed and Pho2–Engrailed A7R. EMSAs were performed with 5-fold serial dilutions of purified protein ranging in concentration from 1.8×10^{-7} M to 1.4×10^{-9} M and radiolabeled UASp1.

between the two proteins (Fig. 1). As predicted, the Pho2–α2 hybrid protein has very little activity at the Pho2-dependent reporters (Table 3). However, we observed that the Pho2–α2 hybrid activates transcription of the endogenous *PHO5* and the UASp1 reporter better than the expression mediated by the Pho2–Engrailed hybrid. Sequence comparison of the Pho2 and α2 HDs reveals that besides the absolutely conserved N51 residue, the only other position which makes a base-specific contact in α2 and that is conserved between the two proteins is R7 (Fig. 1). In the α2 co-crystal structure this residue is making base-specific contacts in the minor groove and it seems likely that residue R7 of Pho2 may be making similar contacts at the UASp1 site (20). We have tested this prediction by making a R7A substitution in the context of the Pho2–α2 chimera. This mutant protein fails to activate transcription of the UASp1 reporter (Table 3). These results show that the R7 residue has an important role in determining the specificity of binding at the UASp1 site.

To determine if the transcriptional activation by the Pho2 chimeras correlate the differences in DNA-binding affinity and specificity, purified hybrid proteins were assayed in EMSAs with radiolabeled UASp1 and UAS_{HIS4}. The Pho2–Engrailed binding affinity to UASp1 is >40-fold weaker than the binding affinity of the wild-type protein (Fig. 5). On the other hand, the binding affinity of the Pho2–Engrailed A7R mutant is at least 5-fold stronger than the Pho2–Engrailed fusion protein to the site. As expected from the results on the expression of the UAS_{HIS4} reporter, Pho2–Engrailed and the Pho2–Engrailed A7R mutant bind with approximately the same affinity to the UAS_{HIS4} site (data not shown). Taken together, these results clearly demonstrate the importance of R7 in the N-terminal arm of Pho2 and support a model where the Pho2 HD N-terminal residues play a more critical role in DNA recognition at *PHO5* than at the *HIS4* and *ADE1* promoters.

DISCUSSION

The co-crystal structure of the Engrailed HD bound to DNA, along with mutagenic studies which have examined DNA-binding specificity of the HD, has provided a detailed model of how the Engrailed HD contacts DNA *in vitro* (19,21,22). We have used this information as a basis to systematically examine the contributions of individual residues of a similar HD protein, Pho2, on transcriptional regulation *in vivo* and DNA-binding affinity *in vitro*. In general, the results agree with our predictions of the Engrailed co-crystal structure and with previous biochemical studies on the protein (19,21,22). For example, alanine substitution of Pho2 residues W48 or N51 result in almost a complete loss of expression from the Pho2-dependent reporter promoters and a 100-fold decrease in the DNA-binding affinity of the proteins. These results are consistent with the observation that residue W48, which is part of the hydrophobic core of the protein, and residue N51, which makes a bidentate contact to an adenine, are conserved in >95% of all HD proteins (1,2). Our results further show that these side chains are important components of HD structure and function *in vivo* and *in vitro*.

It should be noted that, with the exception of the N51A mutant, the R53A substitution has a larger effect on transcriptional regulation and DNA-binding affinity than substitution of any other residue which makes base-specific or phosphate-backbone contacts. This position is conserved in >95% of all HD proteins, and this side chain appears to make a direct contact to at least one, and often two, phosphate groups in the DNA backbone in every HD structure that has been examined (19,20,38–42). Perhaps equally important is that this side chain is often involved in a network of hydrogen bonds with other amino acid side chains and water molecules, which in turn make contacts to the DNA. In addition, the contacts made by this residue may play an important role in positioning the recognition helix of the HD within the major groove. Removal of this side chain may, therefore, disrupt several contacts with the DNA, which would explain why the alanine substitution causes such a large decrease in the level of Pho2 DNA-binding affinity and transcriptional activation.

Although the strong effects of the W48A, N51A and R53A substitutions agree with predictions based on the Engrailed and other HD structures, the effects of substitutions at other positions, which are also strongly conserved and contact the DNA in many HD structures, are relatively weak. For example, alanine substitution of residues T6 or K55, which make contacts to the DNA sugar–phosphate backbone in many HD structures, have relatively little effect on Pho2 DNA-binding affinity or transcriptional activation. These substitutions also only have small effect on the DNA-binding affinity of the Engrailed HD. This result suggests that while these side chains may contact the DNA, they only make a small contribution to the overall DNA-binding affinity of the protein. The small contribution by these side chains may be in part compensated by additional contacts with the DNA by other side chains in Pho2 that are not frequently implicated in DNA binding by HD proteins. For example, we have shown that residues R2, K4 and K58 appear to make some contribution to the activity of the protein. In some of the HD structures, the position of these residues are not well defined (19,20,40–42). However, in the Prd-Gln50 co-crystal structure, the R2 side chain is making a base-specific contact in the minor groove (39) and it is possible that Pho2 makes a similar contact in binding to the UASp1 site. Alternatively, these side

chains may not directly contact DNA, but may play a role in stabilizing the configuration of the N-terminal arm. This would be similar to the structure of the N-terminal arm of α2, which in complex with the **a**1 protein folds back upon itself to make contacts in the minor groove (42). Although the contribution of each individual side chain to the overall binding affinity is relatively weak, in total these side chains are presumably an important component of Pho2 DNA-binding affinity.

We have shown that the Engrailed HD can substitute for the Pho2 HD in activation of UAS_{HIS4} reporter, while the Pho2–Engrailed A7R mutant also functions to activate the expression of the *PHO5* and UASp1 reporters. These results suggest that, with the exception of residue R7 in Pho2, the Pho2 and Engrailed HDs contain the same functional information and are making similar contacts with the DNA. Therefore, it appears that the main difference between the DNA-binding specificity of the Engrailed and Pho2 HDs are due to differences in the N-terminal arm of the proteins. Our results show that the single substitution of A7R in the Engrailed HD changes or relaxes the target specificity of the Pho2–Engrailed hybrid protein so that it will bind to the UASp1 site and properly regulate Pho2-dependent transcription. The importance of residues in the N-terminal arm for DNA-binding and functional activity have been shown for many HD proteins (37,43–52). For example, it has been shown that substitutions of six residues in the N-terminal arm are sufficient to switch the target specificity of the *Drosophila* HD proteins from Deformed to Ultrabithorax (50). However, since residues R3 and R5 are conserved in both of these proteins and are modeled to contact the DNA, it was suggested that the changes in functional specificity may be due to differences in protein–protein interactions rather than in changes of DNA-binding specificity. In the case of the Pho2–Engrailed swap, we show that a change of a single residue in the arm not only changes the functional specificity *in vivo*, but also changes the DNA-binding specificity *in vitro* since the substitution enables the protein to bind to the UASp1 Pho2 recognition site (Fig. 5). These results underscore the importance of residues in the N-terminal arm of HD proteins and their contribution to the DNA-specificity.

The effects of substitutions of residues in the Pho2 N-terminal arm also suggest that the HD contacts DNA in different ways which are dependent on the UAS element. As shown in Table 2, the R5A and R7A substitutions result in minimal activity at *PHO5* and UASp1 while retaining ~50% of wild-type activity at the UAS_{HIS4} reporter construct. This indicates there are different requirements for these side chains at the different promoters. Residue R5 is found in >95% of HDs (1). Mutational analysis, along with structural studies, has shown that this side chain plays an important role in DNA binding by the Engrailed HD (19,21,22). On the other hand, residue 7 is considerably less conserved among HD proteins and in most HD structures this side chain does not appear to be directly involved in contacting the DNA (2,19,38–41,53). This position however does play an important role in influencing differences in the DNA-binding specificity of the *Drosophila* Ubx and Abd-b proteins (46) and has been shown to have an essential role for DNA binding by the yeast α2 HD protein (20,31,42). The differences in the effects of the R5A and R7A substitutions in Pho2 function is likely to be in part due to different DNA sequence requirements at each site, since the R5A protein binds with moderate affinity to the UAS_{HIS4} site but fails to bind to the UASp1 site (Fig. 4). It appears that these contacts by the N-terminal arm have a more important role at the UASp1 sites than at the UAS_{HIS4} site. It is possible that the contacts made by Pho2 in the major groove of the *ADE1* and UAS_{HIS4} elements are more optimal than the contacts made to UASp1, and therefore, Pho2 binding to these sites does not require the contribution of the contacts made by residues in the N-terminal arm. Pho2 binding to UASp1, which may not make optimal contacts in the major groove, may therefore, rely more heavily on contacts made by the N-terminal arm in the minor groove. These results provide an example of how a single HD protein can act to regulate many genes by utilizing its residues, particularly those in the N-terminal arm, to possibly make different sets of contacts with the DNA.

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