Mutations in the Pho2 (Bas2) Transcription Factor That Differentially Affect Activation with Its Partner Proteins Bas1, Pho4, and Swi5*

Leena T. Bhoite‡‡, Jason M. Allen‡‡, Emily Garcia**, Lance R. Thomas‡‡‡, I. David Gregory‡‡‡‡, Warren P. Voth‡, Kristen Wheelihan‡, Ronda J. Rolfes‡, and David J. Stillman‡‡‡‡

From the ‡Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, Utah 84132 and the ‡‡Department of Biology, Georgetown University, Washington D. C. 20057

The yeast PHO2 gene encodes a homeodomain protein that exemplifies combinatorial control in transcriptional activation. Pho2 alone binds DNA in vitro with low affinity, but in vivo it activates transcription with at least three disparate DNA-binding proteins: the zinc finger protein Swi5, the helix-loop-helix factor Pho4, and Bas1, an myb-like activator. Pho2 + Swi5 activates HO, Pho2 + Pho4 activates PHO5, and Pho2 + Bas1 activates genes in the purine and histidine biosynthesis pathways. We have conducted a genetic screen and identified 23 single amino acid substitutions in Pho2 that differentially affect its ability to activate its specific target genes. Analysis of the mutations suggests that the central portion of Pho2 serves as protein-protein interactive surface, with a requirement for distinct amino acids for each partner protein.

Eukaryotic genomes encode a large number of DNA-binding factors. There are 168 and 104 genes encoding homeodomain DNA-binding proteins in humans and Drosophila, respectively, and 607 and 232 genes for zinc finger DNA-binding proteins in these two organisms (1). However, many of these proteins contain very similar DNA-binding domains and may recognize the same DNA sequence in vivo. The Drosophila homeodomain proteins have been well analyzed, and although genetic studies show that these proteins activate different sets of genes in vivo, in vitro DNA-binding experiments show that many of these proteins recognize very similar DNA sequences (2). This apparent conundrum has led to the view that regulatory specificity results from increased DNA binding specificity through cooperative interactions between multiple transcription factors. Cooperativity between transcription factors acts positively to increase affinity and specificity in promoter site recognition (3). Combinatorial mechanisms also allow a transcription factor to act at different genes by interacting with multiple distinct partners.

The yeast PHO2 gene (also known as BAS2 or GRF10) encodes a homeodomain transcription factor that activates transcription in a combinatorial manner. It appears that Pho2 does not act alone, but with several partner proteins. By itself, Pho2 binds DNA with low affinity (4) and comparison of in vitro binding sites reveals a relatively nonspecific consensus, ATTAn TAAT (4–6), similar to the consensus for Drosophila homeodomain proteins (2).

Pho2 binds DNA cooperatively with the Pho4 helix-loop-helix factor to activate expression of the PHO5 acid phosphatase (7). Similarly, Pho2 and the Swi5 zinc finger protein bind cooperatively to the HO promoter and contribute to its transcriptional activation (4, 8, 9). Bas1, an myb-like transcription factor, works with Pho2 in the activation of a set of biosynthetic genes, including HIS4, ADE1, and ADE5,7 (5, 10–12). In vitro studies have not shown cooperative DNA binding between Bas1 and Bas2 (13), however, these experiments were conducted with in vitro expressed proteins that may not reflect their native state in vivo (14). Additionally, two-hybrid analyses detect an interaction between Pho2 and each of its partners, Pho4, Bas1, and Swi5 (9, 15–17), and Pho2 Bas1 interaction has been demonstrated by co-immunoprecipitation (18).

Previous experiments have identified specific amino acids in Swi5 that are important for cooperativity with Pho2, because Swi5 mutant alleles with substitutions at these residues are defective in both cooperative DNA binding and transcriptional activation (9). In this report we describe a reciprocal but expanded strategy to identify Pho2 amino acid residues with discrete roles in activation of a subset of its target genes due to specific defects in individual partner protein interactions.

EXPERIMENTAL PROCEDURES

Strains—The strains used in this study are listed in Table I. Strains DY1921, DY2187, and DY5075 are isogenic strains in the W303 strain background. The HO Site Bi-CYC1::lacZ reporter integrated at the UR-3 locus has been described previously (9), except that in DY5075 LEU2 was inserted into UR3 using plasmid pUL19 (19), a ura3-3 LEU2 converter. Strains RR87 and RR88 are isogenic in the S288C background and have been previously described (15).

Plasmids—The plasmids used in this study are listed in Table I, except for the plasmids with PHO2 point mutations, which are listed in Table II. Plasmid M3570 contains a HindIII fragment with PHO2 cloned into a pRS316 (YCp-URA3) derivative that lacks the BamHI site in the polylinker. M3589 was constructed from M3570 by site-directed mutagenesis with primers F471 (CAATACAGGGATCCCCAC) and F470 (TTCAGGATCCCTTCCC) to introduce BamHI sites that make conservative amino acid substitutions (F273L and A422G). Thus, plasmid M3589 has two BamHI sites, at amino acids 273 and 442 of the
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RESULTS

A Screen for Pho2 Mutants

Strain DY5075 was designed to allow simultaneous screening of PHO2 mutations that affect activation of specific target genes, HO, HIS4, and PHO5. In addition to a PHO2 gene disruption, the strain has an integrated HO(Site B)-IacZ reporter, which contains the Spt15 binding site from the Site B region of the HO promoter acting as a UAS driving expression of lacZ. This reporter turns blue in colonies of a SWI5 PHO2 null mutant strain carrying the URA3 reporter (YRp-TRP1) (15). Amino acids 77–136 comprise the Pho2 homeodomain DNA-binding domain (see Fig. 1), and a large C-terminal region was shown to be required for interaction with Swi5 in vitro. To more precisely identify the regions of Pho2 that interact with its partner proteins, we decided to isolate Pho2 point mutations that interfered with its ability to activate specific target genes. Our strategy combined PCR-mediated random mutagenesis with plasmid gap repair (26) to generate a mutagenized plasmid library of PHO2.

A fragment of Pho2 encoding amino acids 171–504 was subjected to error-prone PCR (Fig. 1). The PCR product was then co-transformed into yeast along with plasmid M3589 that had been cleaved with BamHI. This digestion creates a linearized yeast plasmid with a gap between codons 273 and 422 (Fig. 1). In vivo recombination using the homology between the PCR product and the linearized plasmid repairs the gap and allows formation of a circular plasmid, whereas the URA3 marker on the plasmid allows for selection of these events. Co-transformation of DY5075 with the PHO2 PCR product and linearized M3589 resulted in Ura+ transformants that were screened for either decreased Blue color on X-gal (the White phenotype), reduced growth on media lacking histidine (the His- phenotype), or reduced growth on media lacking inorganic phosphate (the Pho- phenotype). Any mutant showing either a White, His-, or Pho- phenotype was re-screened by the original assay and screened by the other two phenotypic assays. Clearly, a vast majority of these PHO2 mutations were characterized as White, His-, or Pho- phenotypes and subsequently discarded as potential null mutants. We only retained mutants where activation of at least one of the reporters was greater than a pho2 null mutant strain carrying the URA3 vector.

A total of 12,000 yeast transformants containing plasmids bearing potential mutations in Pho2 were screened, and 290 candidates with either decreased expression of the HO(Site B)-IacZ reporter, decreased growth on -His plates, or decreased growth on -Pho plates were identified. After DNA purification, re-transformation and testing in vivo phenotypes, 40 were subjected to DNA sequence analysis. Some clones had single mutations, whereas other clones had multiple amino acid substi-

1 The abbreviations used are: X-gal, 5-bromo-4-chloro-3-D-galactopyranoside; ANOVA, analysis of variance.
Pho2, the B)-lacZ reporter activated by Swi5 and HOthree Pho2-dependent genes, the PHO5 plasmid. rekombination resulting in a circular vivo acids 171 to 326 is PHO2 with seven of the mutations listed. The of the Pho2 point mutations are marked, from 286 to 326 is activation domains. The acidic region matically, showing the DNA-binding and A.-plasmid. B, the DY5075 strain contains three Pho2-dependent genes, the HO/Site B)-lacZ reporter activated by Swi5 and Pho2, the HIS4 gene activated by Bas1 and Pho2, and the PHO5 gene activated by Pho4 and Pho2.

tutions. Among the group with multiple mutations, clones with single amino acid substitutions were generated either by subcloning or by site-directed mutagenesis. We created a set of 23 YCp-URA3 plasmids, each with a single amino acid substitution in PHO2.

Pho2 Mutation Sites and Protein Levels

Although a 334-amino acid region in the center of the protein from 171 to 504 was subjected to mutagenesis, we observed that the mutations tended to fall in two regions (Fig. 1). The first region of ~30 residues was defined by four mutations in positions 244–270, and the second region of ~50 residues was defined by 16 mutations in positions 343–390. Two mutations are on the edge of an acidic patch from 286 to 326 that lies between the two clusters of mutations, and one mutation is found at position 415 in a region implicated in activation function (15).

Western immunoblots were performed to determine whether the point mutations in Pho2 affected protein levels. Yeast strain DY5075 was transformed with plasmids containing the various Pho2 mutants, the wild-type PHO2 gene or the vector control. Extracts were prepared from log phase cells and size-separated on SDS gels for immunoblot analysis (Fig. 2). The blots were probed with antibody specific to Pho2, as well as with as an antibody to Pkp1 as an internal loading control. The results were quantified and are listed in Fig. 2. Most of the Pho2 mutants are expressed at normal levels (defined as expressing between one-fourth to 2-fold of wild-type), with the exception of L244S, F258L and F259L, which showed a severe reduction in Pho2 protein levels (~5%), and F270L and H390L, which showed a strong reduction (10–15%). With the exception of H390L, all of the mutations that exhibit a strong or severe decrease in Pho2 protein levels are found in a cluster of mutation amino-terminal to the acidic patch. Because these mutations have a strong effect on Pho2 protein levels, and most likely can account for the decrease in gene expression detected, we have largely excluded them from the analyses below.

Analysis of Pho2 Point Mutants

Pho4-dependent Expression—The secreted acid phosphatases are encoded by three genes, PHO5, PHO11, and PHO10 (27). The better studied of these, PHO5, encodes the major form, whereas the other two genes encode minor species. The expression of these genes is repressed when cells are grown in medium containing inorganic phosphate, and derepression requires the activity of Pho4 and Pho2 (27). To determine the ability of the Pho2 mutants to activate expression of these genes, we measured acid phosphatase activity in cellular extracts (Table II) and normalized the expression data to both wild-type expression and Pho2 protein levels. Ten of the remaining eighteen mutations greatly affected expression of the secreted phosphatases (less than 26% activity remaining). These ten mutations are found tightly clustered between positions 347 and 378 and exhibit phosphatase activities that are undetectable and up to 20% of the wild-type. Phosphatase expression was affected in strains with the mutations F347L, V349G, C369Y, D370G, D371G, D371N, F372L, E374G, E374V, and V378G. Interestingly, each of these mutations also affect expression of the Swi5- and Bas1-dependent reporters (described below), indicating that these mutations either alter the overall structure of the region, or affect critical contacts required for all three partners. We did not identify any Pho2 alleles that specifically diminished phosphatase expression (Pho4-dependent) while retaining expression of the other reporters.

Swi5-dependent Expression—Strain DY5075 contains the integrated HO/Site B)-lacZ reporter, the expression of which is dependent on Swi5 and Pho2. We next determined the effect of the PHO2 mutations on expression of this reporter. Extracts were prepared from log phase cells and used for quantitative β-galactosidase assays (Table II). β-Galactosidase activities are presented as specific activities and as a percentage normalized to the PHO2 wild-type and Pho2 protein levels. Fourteen mutations in PHO2 affected expression of this reporter leading to β-galactosidase activities that were less than 26% of wild-type.
Pho2 protein amounts, normalized to Pgk1, are given in the figure as a control. The Pho2 and Pgk1 protein levels were quantitated, and the stripped, and then probed with antibody to Pgk1 as an internal loading control. The Western blots were probed with polyclonal antibody to Pho2 to measure Pho2 protein levels, on SDS gels and transferred to nitrocellulose. The Western blots were expression of I388V, and I415V. The N363S mutation strongly affected expression. Strikingly, mutations in the adjacent residues N387S, K331G, and R343K appear to be specific for Bas1. The Pho2 mutations affect gene expression above background.

Bas1-dependent Expression—Almost twenty genes require the combination of Bas1 and Pho2 for expression, including genes required for metabolism of histidine, purine, and pyrimidine nucleotides and one-carbon units (5, 10–12). The HIS4 gene is activated by one of two pathways, either by Pho2 in combination with Bas1 or by Gcn4 acting alone (5). The ADE5,7 gene is activated by Bas1 and Pho2 but does not require Gcn4 (10, 28). The HIS4 and ADE5,7 genes also differ in their dependence on Pho2 in an assay based on activation by Bas1-VP16 (17); HIS4 expression is strongly dependent on Pho2, whereas ADE5,7 exhibits a moderate requirement. Several other ADE genes, typified by ADE1, have only a weak dependence on Pho2 (17).

To determine the ability of the Pho2 mutants to activate HIS4 expression, a gcn4 pho2 strain was transformed with a HIS4-lacZ reporter plasmid and the set of Pho2 plasmids. Extracts were prepared from log phase cells and used for quantitative β-galactosidase assays (Table III). β-Galactosidase activities are presented as normalized to the PHO2 wild-type and Pho2 protein levels. Eleven mutations exhibited substantial decreases in expression of HIS4-lacZ, largely overlapping with the ones described above that affect phosphatase expression. The Pho2 mutations R343K, F347L, V349G, N363S, C369Y, D370G, F372L, V378G, and I415V (but not the mutations at E374) strongly affected HIS4-lacZ expression. In addition to these mutations, R343K is strongly decreased for HIS4 expression, expressing only 2% of the wild-type level, and two mutations, D319A and K331G, appear to have completely lost the ability to activate HIS4 expression with Bas1, exhibiting empty-vector levels of β-galactosidase activity. These mutations are located within or immediately adjacent to the acidic region (positions 286–326). Finally, the I388V mutation described above as showing a strong decrease in expression of HO(Site B)-lacZ when paired with Swi5, exhibited a 3.25-fold increase in HIS4-lacZ expression relative to WT Pho2. Thus, mutations D319A, K331G, and R343K appear to be specific for Bas1.

To determine the ability of the Pho2 mutants to activate ADE5,7 expression, an ADE5,7-lacZ reporter plasmid and the set of Pho2 plasmids were transformed into a pho2 strain (RR87). Cells were grown in the absence of added adenine to induce expression of ADE genes, and extracts were prepared for quantitative β-galactosidase assays (Table III). β-Galactosidase activities are presented as normalized to the PHO2 wild-type and Pho2 protein levels. In general, the same set of mutations that affected HIS4-lacZ expression also affected expression of ADE5,7-lacZ except for the three Bas1-specific alleles, D319A, K331G, and R343K, which had essentially normal levels of ADE5,7-lacZ expression. The finding that these latter three alleles exhibited a significant decrease only at HIS4-lacZ could be a result of HIS4 having a strong requirement for Pho2 (17) or that strain background differences affect expression. The D371G mutation, which was only mildly affected for HIS4-lacZ expression, did not promote ADE5,7-lacZ expression above background.

One-hybrid Assay for Interaction—The analysis of the Pho2 mutations described above has revealed two alleles specific for Swi5 (N387S and I388V) and three alleles that affect expression of one or both of the Bas1-dependent reporters (D319A and K331G). Because the Pho2 mutations affect gene expression but do not co-localize with the DNA-binding domain or the...
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For acid phosphatase assays, plasmids were transformed into DY1921 (pho2) and grown in medium lacking inorganic phosphate and assayed for acid phosphatase activity. For HO/Site B-lacZ reporter assays, plasmids were transformed into BY5075 (ace2 pho2 gcn4 ura3::LEU2::HO Site B-CYC1-lacZ), and transformants were grown in medium lacking racil and assayed for β-galactosidase activity. Three independent transformants were assayed, and standard errors are shown. After subtracting the value assayed in the pho2 (vector), the normalized levels are also shown as a percentage of WT and normalized to the protein levels quantitated from Fig. 2.

| Pho2 mutant | Acid phosphatase | HO/Site B-lacZ |
|-------------|------------------|---------------|
| Substitution | Plasmid | Raw value | Normalized to | Raw value | Normalized to |
| Vector       | pRS316          | 0.04 ± 0.01 | 0 | 0 | 0.03 ± 0.03 | 0 |
| WT Pho2      | M3570           | 0.66 ± 0.08 | 100 | 100 | 360 ± 40 | 100 |
| D319A        | M4117           | 0.81 ± 0.08 | 120 | 167 | 400 ± 45 | 149 |
| K331G        | M4118           | 0.59 ± 0.04 | 89 | 136 | 415 ± 75 | 120 |
| R343K        | M3919           | 0.15 ± 0.04 | 17 | 54 | 60 ± 6 | 15 |
| F347L        | M4122           | 0.06 ± 0.01 | 2 | 3 | 28 ± 2 | 6 |
| V349G        | M3921           | 0.03 ± 0.01 | >0 | >0 | 34 ± 0.2 | 7 |
| N363S        | M4123           | 0.11 ± 0.02 | 11 | 33 | 24 ± 3.8 | 5 |
| S367A        | M3923           | 0.71 ± 0.02 | 109 | 93 | 370 ± 20 | 100 |
| C369Y        | M4124           | 0.11 ± 0.01 | 10 | 20 | 25 ± 3 | 5 |
| D370G        | M4125           | 0.07 ± 0.01 | 5 | 7 | 15 ± 4 | 2 |
| D371G        | M3922           | 0.08 ± 0.01 | 6 | 20 | 8 ± 0.6 | 0 |
| D371N        | M4126           | 0.08 ± 0.02 | 6 | 15 | 15 ± 1 | 2 |
| F372L        | M3924           | 0.07 ± 0.01 | 4 | 8 | 15 ± 3 | 2 |
| E374G        | M4129           | 0.16 ± 0.01 | 19 | 20 | 32 ± 9 | 7 |
| E374V        | M4131           | 0.05 ± 0.03 | 1 | 1 | 21 ± 7 | 4 |
| V378G        | M4119           | 0.05 ± 0.01 | 1 | 2 | 32 ± 3 | 7 |
| N387S        | M4133           | 0.35 ± 0.03 | 46 | 52 | 88 ± 4 | 26 |
| E388V        | M4134           | 0.50 ± 0.12 | 73 | 67 | 89 ± 8 | 20 |
| I415V        | M4135           | 0.38 ± 0.07 | 55 | 34 | 93 ± 45 | 24 |

| HI54-lacZ | AD5E5,7-lacZ |
|-----------|--------------|
| Pho2 mutant | Raw value | Wild-type | Protein | Raw value | Wild-type | Protein |
| Vector     | pRS316      | 5 ± 1    | 0       | 0        | 14 ± 5   | 0        |
| WT Pho2    | M3570       | 120 ± 24 | 100     | 100      | 87 ± 34  | 100      |
| D319A      | M4117       | 2.0 ± 0.3| >0      | >0       | 47 ± 11  | 62       |
| K331G      | M4118       | 2.7 ± 0.2| >0      | >0       | 55 ± 16  | 77       |
| R343K      | M3919       | 5.5 ± 1.7| 0       | 2        | 34 ± 24a| 38       |
| F347L      | M4122       | 6.5 ± 0.9| 1       | 2        | 15 ± 5b | 2        |
| V349G      | M3921       | 7.1 ± 2.2| 2       | 4        | 24 ± 3b | 19       |
| N363S      | M4123       | 15 ± 3   | 9       | 26       | 14 ± 3b | 0        |
| S367A      | M3923       | 105 ± 70 | 87      | 75       | 41 ± 6a | 51       |
| C369Y      | M4124       | 6 ± 2    | 1       | 2        | 17 ± 4b | 6        |
| D370G      | M4125       | 21 ± 4   | 14      | 22       | 16 ± 4b | 4        |
| D371G      | M3922       | 17 ± 2   | 10      | 38       | 14 ± 3b | 0        |
| D371N      | M4126       | 64 ± 39  | 51      | 126      | 23 ± 6b | 17       |
| F372L      | M3924       | 63 ± 1.3 | 1       | 3        | 15 ± 4b | 2        |
| E374G      | M4129       | 152 ± 22 | 130     | 143      | 34 ± 4a | 38       |
| V378G      | M4131       | 83 ± 10  | 68      | 69       | 48 ± 7  | 64       |
| N387S      | M4133       | 16 ± 3   | 10      | 24       | 11 ± 3  | ND       |
| E388V      | M4134       | 85 ± 7   | 70      | 80       | 41 ± 8b | 51       |
| I415V      | M4135       | 410 ± 90 | 350     | 325      | 76 ± 25 | 120      |

* P < 0.01.
* P < 0.001.
* ND, not determined.

To test our Pho2 mutants for the ability to interact with Bas1 with this assay, the set of plasmids with Pho2 mutations was transformed along with a lexAop-lacZ reporter plasmid into strain RR88 (pho2 bas1-2) expressing the LexA-Bas1 fusion protein. Cells were grown in the absence of added adenine, which stimulates the Bas1-Pho2 interaction, and extracts were prepared for quantitative β-galactosidase assays (Table IV). β-Galactosidase activities are presented as normalized to the activation domain, we postulate that these mutations affect interaction with partners. One test for partner interaction is based on a one-hybrid assay using a LexA-Bas1 fusion protein (15). In these experiments, LexA-Bas1 activates expression of a lexAop-lacZ reporter, but this activation sharply decreases in the absence of Pho2. Thus, the activation by LexA-Bas1 is dependent upon Pho2, but the Pho2 DNA-binding domain is not required for this stimulation.
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For the LexA-Bas1 activation assays, the Pho2 plasmids (URA3 marker) were transformed into RR88 (pho2 bas1) along with p2099, the LexA-Bas1 plasmid (HIS6 marker), and pH335, the lexAop-lacZ reporter (TRP1 marker). Transformants were grown in medium lacking uracil, histidine, tryptophan, and adenine, and assayed for β-galactosidase activity. At least three independent transformants were assayed and standard errors are shown. ANOVA analysis was performed to determine the probability that the activity measured was different than the level expressed by the WT Pho2 protein. After subtracting the values assayed in the pho2 (vector), the normalized levels are also shown as a percentage of WT and normalized to the protein levels quantitated from Fig. 2.

### TABLE IV

One hybrid interaction: lexAop-lacZ reporter assays

| Pho2 mutant | LexA-Bas1 activation | Normalized | Normalized to protein level |
|-------------|----------------------|------------|-----------------------------|
| Vector      | 1300 ± 540           | 0          | 0                           |
| WT Pho2     | 3360 ± 1450          | 100        | 100                         |
| D319A       | 2250 ± 330           | 46         | 62                          |
| K331G       | 5140 ± 220           | 190        | 285                         |
| R343K       | 1960 ± 540           | 32         | 100                         |
| F347L       | 2000 ± 310           | 34         | 52                          |
| V349G       | 1540 ± 190a          | 12         | 25                          |
| N363S       | 1500 ± 380a          | 10         | 29                          |
| S367A       | 1340 ± 620a          | 2          | 2                           |
| C369Y       | 1670 ± 350a          | 18         | 36                          |
| D370G       | 2620 ± 230           | 64         | 96                          |
| D371G       | 1640 ± 300a          | 17         | 57                          |
| D371N       | 2810 ± 750           | 78         | 190                         |
| F372L       | 3100 ± 770           | 87         | 180                         |
| E374G       | 6000 ± 1900b         | 230        | 251                         |
| E374V       | 3160 ± 420           | 90         | 91                          |
| V375G       | ND                   | ND         | ND                          |
| N387S       | 1290 ± 410b          | >0         | >0                          |
| I387V       | 4000 ± 540           | 130        | 120                         |
| I145V       | 2760 ± 920           | 71         | 45                          |

-<sup>a</sup> P < 0.01.
-<sup>b</sup> ND, not determined.

**PHO2** wild-type and Pho2 protein levels. Four mutations showed substantially reduced activation of the reporter by LexA-Bas1, suggesting that these Pho2 mutants are defective in Bas1 interaction, or in activation *per se*. Interestingly, the V349G mutation strongly affects HIS4-lacZ expression with only a modest effect at ADE5,7-lacZ, whereas the N363S shows the opposite pattern; both of these mutations exhibited about the same level of lexAop-lacZ expression. Interestingly, two Pho2 alleles, S367A and N387S, exhibited a severe defect in activating the lexAop-lacZ reporter (<2% expression) but they exhibited only mild effects on the HIS4-lacZ and ADE5,7-lacZ reporters. In general, this assay appeared to better correlate more closely with the expression pattern from the ADE5,7-lacZ reporter, rather than the HIS4-lacZ reporter. Two alleles, K331G and E374G, exhibited a greater than 2-fold increase in expression, which did not reflect the expression patterns of either HIS4-lacZ or ADE5,7-lacZ. Finally, this assay failed to detect the strongly decreased expression seen at both HIS4-lacZ and ADE5,7-lacZ with the Pho2 alleles F347L, D370G, and F372L.

We have conducted three assays to examine how the Pho2 mutations affect interaction with Bas1, at HIS4-lacZ and ADE5,7-lacZ with native Bas1 and at lexAop-lacZ with LexA-Bas1. In general, the three assays gave similar results for the twenty-two mutations, although specific differences were detected (D319A and K331G at HIS4-lacZ versus S367A and N387S at lexAop-lacZ). Although it may be possible that differences in the genetic background or growth conditions affected our interpretation, an intriguing possibility is that the expression differences among the Pho2 mutant alleles reflect the different binding contexts with Bas1.

**FIG. 3.** Effect of Pho2 mutations on gene expression. The normalized data from Tables II and III are represented in graphical format. The L244S, F258L, F259L, and F270L mutations that affect protein levels are not included.

**DISCUSSION**

The Pho2 protein provides a model for combinatorial control of transcriptional regulation. Pho2 binds DNA very poorly on its own but works with three distinct partner proteins, the Swi5 zinc finger protein, the Pho2 helix-loop-helix protein, and the Bas1 myb-like protein. In this report we identify and characterize Pho2 point mutations that selectively affect the ability of Pho2 to activate transcription with different partner proteins.

We previously conducted a deletion analysis of Pho2 to determine what regions are required for cooperative DNA-binding *in vitro* with Swi5 (25). These studies showed that the DNA-binding domain (amino acids 77–136) of Pho2 is not sufficient for cooperative DNA binding and that a large C-terminal region was required for interaction with Swi5 *in vitro*. Interestingly, truncations that remove part of this region are unstable in *E. coli*, and these C-terminal truncation polypeptides expressed from an *in vitro* transcription/translation system are unable to even bind DNA, despite the fact that the homeodomain DNA-binding region is intact. These results suggest that this C-terminal region of Pho2 comprises a specific protein domain and that deletion endpoints within this region cause aberrant protein folding. Studies by Berben et al. (29) demonstrated that a C-terminal truncation of residues past 407 is still functional *in vivo* for PHO5 activation, whereas a truncation of residues before 404 is completely inactive. We conclude from these studies that the domain of Pho2 required for partner interaction is within amino acids 170–407. Importantly, the point mutations we obtained, which selectively affect activation of reporters with the Swi5, Pho2, and Bas1 partner proteins, lie within this region. These results are consistent with two-hybrid experiments showing that amino acids 112–404 of Pho2 are required for interaction with Bas1 (18).

Fig. 3 compares the effect of the Pho2 point mutations on expression of HO-lacZ, acid phosphatase, HIS4-lacZ, and ADE5,7-lacZ. Several mutations (L244S, F258L, F259L, and F270L) are not considered in Fig. 3, because they strongly affect protein levels (see Fig. 2). Three of these substitutions are a relatively conservative change of phenylalanine for leucine, but they imparted a severe effect on protein stability. Considering this observation in light of the stability and folding problems discussed above for truncations expressed in *E. coli* and *in vitro*, these findings suggest that the correct folding of this region requires contributions by very specific amino acids.
expression of the reporter genes. Notably, within this region is a set of tightly clustered mutations. C369Y, D370G, D371G, D371N, F372L, E374G, and E374V, that affect five of six positions and perhaps form the core of the interaction region, because the reporters for all partners showed strong effects on expression. A second cluster, positions 387–390, is found just C-terminal to the core. Mutations here show more specificity: N387S is specifically affected for HO(Site B)-lacZ expression, and, interestingly, I388V exhibits a strong decrease in HO(Site B)-lacZ expression but a 3.25-fold increase in HIS4-lacZ expression. A third cluster within this region lies N-terminal to the core at 343–349 and adjacent to the mutations that showed specificity for HIS4-lacZ expression (D319A and K331G). Taken together, the region from 343 to 390 appears to be principally involved in partner interaction, with many amino acid substitutions having general effects and a small number of substitutions having specific affects.

We also were able to investigate effects of the Pho2 mutations with a single partner, Bas1, in three unique contexts, at HIS4-lacZ, ADE5-lacZ, and lexAop-lacZ. Particularly interesting are the mutations D319A, K331G, and R343K, which severely affect HIS4-lacZ expression, and S367A and N387S, which severely affect lexAop-lacZ expression; these five mutants exhibit only mild effects on expression with the other two Bas1-dependent reporters. That we detected strong effects on expression unique for the context suggests that Bas1 and Pho2 do not form the same kind of ternary complex with DNA at each promoter. Although this may be expected when Pho2 forms a ternary complex with the LexA-Bas1 fusion protein, it is interesting that we could detect this context dependence at two reporters using native Bas1. These results fit nicely with the experiment of Pinson et al. (17) who used a Bas1-VP16 fusion protein to investigate the requirement for Pho2/Bas2 at HIS4 and several ADE promoters. In their experiment they showed that Bas1-VP16 strongly required Pho2 to activate HIS4-lacZ expression, it modestly required Pho2 to activate ADE5,7-lacZ, and it had nearly no requirement for Pho2 to activate the other ADE promoters. Our results here also support the findings of Barbaric et al. (7) who showed that the requirement for Pho2 is different at UASp1 of the PHO5 promoter than at UASp2. At UASp1, Pho2 cooperates with Pho4 to allow binding, but at UASp2 Pho2 is required for transactivation by Pho4. Together, these results suggest that Pho2 has different ways to cooperate with each of its partners to determine ternary complex formation and activation function at each unique promoter context.

This analysis has provided a detailed description of mutations in Pho2 that affect its function with three different co-regulators and has provided new insights into the structure of this interesting protein, required for expression of nearly two dozen genes in Saccharomyces. This collection of Pho2 mutants should be a useful set of reagents to further explore the role of protein-protein interactions in combinatorial control of gene expression.

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