REVIEW

The phosphatase system in *Saccharomyces cerevisiae*

Yasuji Oshima

Department of Biotechnology, Faculty of Engineering, Kansai University,
3-3-35 Yamatecho, Suita-shi, Osaka 564, Japan

The yeast *Saccharomyces cerevisiae* has at least six species of acid and alkaline phosphatases with different cellular localizations, as well as inorganic phosphate (P_i) transporters. Most of the genes encoding these enzymes are coordinately repressed and derepressed depending on the P_i concentration in the growth medium. The P_i signals are conveyed to these genes through a regulatory circuit consisting of a set of positive and negative regulatory proteins. This phosphatase system is interested as one of the best systems for studying gene regulation in *S. cerevisiae* due to the simplicity of phenotype determination in genetic analysis. With this methodological advantage, considerable amounts of genetic and molecular evidence in phosphatase regulation have been accumulated in the past twenty-five years. This article summarizes the current progress of research into this subject.

INTRODUCTION

*Saccharomyces cerevisiae* has several phosphatases, with different specificities and cellular locations, and permeases, used in inorganic phosphate (P_i) uptake. The set of genes responsible for these activities is coordinately regulated repressively by P_i concentration in the growth medium. This phosphatase (*PHO*) system, consisting of several genes and regulators (Table 1), has been studied extensively due to numerous technical advantages (Oshima, 1991). Acid phosphatase activity is easily detected for each colony by specific staining, because the enzyme is located on the cell surface. Enzyme synthesis is regulated in response to a simple effector, P_i, in the medium. The same regulatory system (with minor differences) also controls the synthesis of alkaline phosphatase, located in the cell envelope, whose activity is also detectable in colonies by staining after permeabilizing the cells to the substrate. There is a 500-fold difference between the repressed and derepressed levels of acid phosphatase activity. With the above technical easiness in the mutant selection, we were able to isolate various mutants that were deficient in or constitutive for synthesis of repressible acid and alkaline phosphatases. Genetic and the subsequent molecular analysis of the *PHO* system with these mutants over the twenty-five years has provided insight into the mechanism of signal transduction for gene regulation. In this article, I attempt to summarize the current understanding of the molecular mechanism in phosphatase regulation, especially in signal transduction between negative and positive regulators and cis-acting regulatory sites in the *PHO* promoters. For more details on the initial genetic study of the *PHO* system, see review by Oshima (1982), and that by Johnston and Carlson (1992) on the subsequent molecular analysis.

THE GENES AND PROTEINS CONCERNING PHOSPHOROUS METABOLISM

The genes that are regulated by P_i are *PHO5*, encoding the major fraction of repressible acid phosphatase, designated p60 (rAPase; EC 3.1.3.2; Arima et al., 1983; Bajwa et al., 1984; Bostian et al., 1980; Lemire et al., 1985; Toh-e et al., 1975b), *PHO10* (or *PHO12*; de Steensma et al., 1989) and *PHO11* encoding p58 and p56 isozymes of the p60 enzyme respectively (Bostian et al., 1980; Lemire et al., 1985), *PHO8* encoding a non-specific repressible alkaline phosphatase (rALPase; EC 3.1.3.1; Kaneko et al., 1982, 1987; Toh-e et al., 1976), *PHO84* encoding a P_i-transporter (Bunya et al., 1991), and *PHO81* encoding one of the regulatory proteins in the *PHO* system (Ogawa et al., 1993; Yoshida et al., 1989b). These three rAPase isozymes, p60, p58, and p56, (optimum pH 3–4) are glycoproteins (Boer and Steyn-Parve, 1966; Onishi et al., 1979) and located in the cell wall or the periplasmic space (Arnold, 1972; Linnemans et al., 1977; Lopandic et al., 1987) and are probably responsible for acquiring phosphate from diverse extracellular sources. rALPase (optimum pH 8) encoded by *PHO8*, which is also a glycoprotein, is located in the vacuole (Clark et al., 1982) and cleaves diverse substrates to retrieve phosphate from intracellular products. It was suggested that the most important substrate of Pho8p is fructose-2, 6-bisphosphate (Plankert et al., 1991).

*S. cerevisiae* also has other phosphatases; one is an acid phosphatase (T-rAPase), encoded by *PHO3*, which may func-
tion to acquire thiamine (Nosaka, 1990; Schweingruber et al., 1986), and another alkaline phosphatase encoded by *PHO13* exhibiting specificity only for *p*-nitrophenylphosphate (Kaneko et al., 1989). The *PHO3* gene, which is closely linked with *PHO5* on the right arm of chromosome II (Toh-e and Oshima, 1975), is only expressed when *PHO5* is not expressed (Tait-Kamradt et al., 1986) and modestly repressed by thiamine (Nosaka, 1990; Schweingruber et al., 1986) under the positive regulation of the proteins encoded by *PHO6* (= *THI2*) (Nishimura et al., 1992a; Toh-e et al., 1975a) or *PHO7* (Toh-e et al., 1975a) or *THI3* (Nishimura et al., 1992b). The *PHO13* gene is expressed constitutively (Toh-e et al., 1976).

Beside phosphatases, *S. cerevisiae* has at least two systems for P, uptake; one with a low $K_m$ value (8.2 µM) and the other with a high $K_m$ value (770 µM) for external P, (Tamai et al., 1985). The low $K_m$ system is repressed by P, via the same system as that for rAPase regulation and requires the *PHO84* gene product for its function. From the nucleotide sequence of cloned *PHO84*, it was predicted that Pho84p is a 65-kDa 596-amino acid protein homologous to that of several sugar transporters of mammalian, yeast, and bacterial origin (Bun-ya et al., 1991). Pho84p has 12 putative transmembrane segments, each consisting of 21 amino acid residues and the majority of which are hydrophobic. These putative transmembrane segments are separated into two groups by a 74-amino acid segment, which is rich in charged amino acid residues and is located between the sixth and seventh segments. These architectural characteristics and the amino acid sequences of sev-

---

**Table 1. Genes and their encoding proteins or functions in the PHO system**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome location</th>
<th>Function of gene-product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structural genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHO3</td>
<td>2R</td>
<td>Thiamine phosphatase (T-rAPase)</td>
<td>Nosaka (1990)</td>
</tr>
<tr>
<td>PHO5</td>
<td>2R</td>
<td>rAPase, p60</td>
<td>Toh-e et al. (1975b)</td>
</tr>
<tr>
<td>PHO10</td>
<td>8R</td>
<td>rAPase, p58</td>
<td>Bostian et al. (1980)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lemire et al. (1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Venter and Hörz (1989)</td>
</tr>
<tr>
<td>or PHO12</td>
<td>8R</td>
<td></td>
<td>de Steensma et al. (1989)</td>
</tr>
<tr>
<td>PHO11</td>
<td>1R</td>
<td>rAPase, p56</td>
<td>Bostian et al. (1980)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lemire et al. (1985)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Venter and Hörz (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>de Steensma et al. (1989)</td>
</tr>
<tr>
<td>PHO8</td>
<td>4R</td>
<td>rALPase</td>
<td>Kaneko et al. (1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fructose-2, 6-bisphosphate 6-phosphatase</td>
<td>Plankert et al. (1991)</td>
</tr>
<tr>
<td>PHO9 (PEP4)</td>
<td>16L</td>
<td>Protease for Pho8p maturation</td>
<td>Kaneko et al. (1982)</td>
</tr>
<tr>
<td>PHO13</td>
<td>4L</td>
<td>Specific <em>p</em>-nitrophosphatase</td>
<td>Kaneko et al. (1989)</td>
</tr>
<tr>
<td>PHO84</td>
<td>13L</td>
<td>P, transporter</td>
<td>Bun-ya et al. (1991)</td>
</tr>
<tr>
<td>PHO86</td>
<td>4R</td>
<td>P, transporter associate</td>
<td>Yompakdee et al. (1996a)</td>
</tr>
<tr>
<td>PHO87</td>
<td>3R</td>
<td>P, transporter associate</td>
<td>Bun-ya et al. (1996)</td>
</tr>
<tr>
<td>PHO88</td>
<td>2R</td>
<td>P, transporter associate</td>
<td>Yompakdee et al. (1996b)</td>
</tr>
<tr>
<td>GTR1</td>
<td>13L</td>
<td>P, transporter associate</td>
<td>Bun-ya et al. (1992)</td>
</tr>
<tr>
<td><strong>Regulatory genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHO2 (BAS2)</td>
<td>4L</td>
<td>DNA-binding transcriptional activator</td>
<td>Sengstag and Hinnen (1987)</td>
</tr>
<tr>
<td>PHO4</td>
<td>6R</td>
<td>DNA-binding transcriptional activator</td>
<td>Yoshida et al. (1989a)</td>
</tr>
<tr>
<td>PHO6 (THI2)</td>
<td>2R</td>
<td>Required for <em>PHO3</em> expression</td>
<td>Nishimura et al. (1992a,b)</td>
</tr>
<tr>
<td>PHO7</td>
<td>16L</td>
<td>Cyclin; inhibitor of Pho4p</td>
<td>Toh-e and Shimauchi (1986)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kavasaki et al. (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Uesono et al. (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kaffman et al. (1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHO81</td>
<td>7R</td>
<td>Inhibitor of Pho80p/Pho85p</td>
<td>Schneider et al. (1994)</td>
</tr>
<tr>
<td>PHO85</td>
<td>16L</td>
<td>Cyclin-dependent kinase; inhibitor of Pho4p</td>
<td>Uesono et al. (1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Toh-e et al. (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kaffman et al. (1994)</td>
</tr>
</tbody>
</table>
eral segments of Pho84p are highly similar to those of proteins in the human glucose transporter superfamily (Mueckler et al., 1985).

During the sequence determination of PHO84, another open reading frame (ORF) was detected in a cloned PHO84 fragment at a head-to-head position of the PHO84 ORF (Bun-ya et al., 1992). This ORF, designated GTR1, encodes a 310-amino acid protein containing the characteristic tripartite consensus elements for binding GTP, conserved in GTP-binding proteins of the ras family, in its N-terminal region. Disruption of GTR1 resulted in a slow-growth phenotype at 30°C and no growth at 15°C, and conferred the phenotypes of constitutive synthesis of rAPase, reduced P_i uptake activity, and resistance to arsenate (a toxic P_i analog) on the cells, similar to the pho84 mutant. Therefore, Gtr1p encoded by GTR1 might be associated with the P_i transport mechanism.

Two other genes that may be involved in the P_i transport mechanism were detected in a mutant exhibiting arsenate resistance and constitutive synthesis of rAPase (Bun-ya et al., 1996). The mutant was shown to have double mutations designated pho86 and pho87. This double mutant transcribes PHO84 even in a high-P_i, medium but has a severe defect in P_i uptake. The pho86 single mutation conferred a substantial reduction of P_i uptake on the cells, while the pho87 single mutant or a disruptant of it showed no apparent difference in its phenotypes with those of the wild-type cells. Sequence determination of a partial PHO87 coding region revealed that it is identical to YCR524 on the right arm of chromosome III (Thierry et al., 1990). The published nucleotide sequence of YCR524 predicts that PHO87 encodes a 923-amino acid protein with a highly charged N-terminal half and a C-terminal half consisting of twelve transmembrane segments (Bun-ya et al., 1996). The Pho86p protein, deduced from the nucleotide sequence of PHO86, is a 34-kDa membrane protein consisting of 311 amino acid residues with two strongly hydrophobic segments in its N-terminal half (Yompakdee et al., 1996a). Thus, Pho86p and Pho87p are likely to be involved in the P_i transport mechanism along with Pho84p and Gtr1p. Both disruption (Yompakdee et al., 1996a) and increased dosage (Yompakdee et al., 1996b) of PHO86 activates P_i-inhibited Pho81p produced under the control of the GAL1 promoter (described below).

Another putative membrane protein, Pho88p, was identified by screening for a multicopy suppressor of the rAPase phenotype caused by the P_i inhibition of Pho81p (Yompakdee et al., 1996b). The nucleotide sequence of the cloned fragment revealed that the gene, encoding Pho88p, is identical to YBR106w (Feldmann et al., 1994). The hydrophobicity profile of the amino acid sequence predicted from the published nucleotide sequence of YBR106w and the cellular location suggested that Pho88p is a membrane protein. Both disruption and high dosage of PHO88, similarly to PHO86, resulted in the activation of P_i-inhibited Pho81p and reduced P_i uptake. The double disruption of pho86 and pho88 resulted in a somewhat higher level of rAPase activity in a high-P_i, medium and conferred a higher arsenate resistance on the cells than on cells with single disruptants of these genes. These characteristics of Pho88p strongly suggest that it is also involved in P_i uptake and, together with Pho86p, modulates Pho81p activity as a primary receptor for P_i signals from the environment.

**REGULATORY CIRCUITS**

For the regulation of the activities of the above enzymes and the P_i uptake system, a complex interplay between numerous genes and proteins is required. In a current model for phosphatase regulation (Fig. 1), a positive regulator (or positive factor) Pho4p, coded for by PHO4, is indispensable for the transcriptional activation of the above PHO structural genes by binding at a specific cis-acting regulatory site(s), termed upstream activation site (UAS),
in the promoter. An additional DNA binding protein, Pho2p (also known as Bas2p or Grf10p; Yoshida et al., 1989b), is involved in the transcription of all the PHO structural genes except PHO8, but it does not have a direct function in the transduction of P signals. Pho2p activates the transcription of numerous other genes, including HIS4 (Arndt et al., 1987; Tice-Baldwin et al., 1989), TRP4 (Braus et al., 1988), CYC1 (Sengstag and Hinne, 1988), genes for adenine biosynthesis (Arndt et al., 1987; Daignan-Fornier and Fink, 1992), and HO involved in the mating-type-switching mechanism (Brazas and Stillman, 1993a,b) in combination with a cognate positive regulator.

In a high-P, medium, a complex of negative regulators, Pho80p (cyclin) and Pho85p (cyclin-dependent protein kinase), phosphorylates five serine residues of Pho4p (Kaffman et al., 1994). The complex then turns off the transcription of PHO genes by reducing the nuclear localization of Pho4p (O’Neill et al., 1996). When the P, concentration in the medium is sufficiently low, Pho6p and Pho85p may stimulate Pho1p activity (Yampakdee et al., 1996b). Then the activated Pho81p inhibits the function of the Pho80p/Pho85p complex (Ogawa et al., 1995a; Schneider et al., 1994), thus allowing Pho4p, in collaboration with Pho2p, to activate the transcription of the PHO genes (Fig. 2; see review, Lenburg and O’Shea, 1996).

The PHO4 (Yoshida et al., 1989a), PHO80 (Madden et al., 1990), and PHO85 (Madden et al., 1990; Uesono et al., 1992) genes are transcribed constitutively at low levels. PHO2 transcription is also at a low level but is self-regulated (Yoshida et al., 1989a). The transcription of PHO86 is under relaxed regulation of P, while that of PHO87 is constitutive (Bun-ya et al., 1996), and that of PHO88 is not affected by the P, concentration in the medium (Yampakdee et al., 1996b). GTR1 is transcribed at an extremely low level independent of the P, concentration in the medium, while it shares the promoter region with PHO84 but in the reverse direction (Bun-ya et al., 1992). In contrast, the transcription of PHO81, encoding Pho81p, is repressed by P, via the same PHO regulatory system, indicating that the PHO regulatory circuit forms a positive feedback loop (Ogawa et al., 1993; Yoshida et al., 1989b).

Positive regulators Pho2p and Pho4p are DNA binding proteins. The nucleotide sequence of PHO4 suggests that Pho4p consists of 312 amino acid residues (Yoshida et al., 1989a). It has the characteristics of a basic region beside an amphipathic helix-loop-helix (bHLH) structure known as a DNA binding domain and homodimer formation (Murre and Baltimore, 1992) in the 85-amino acid C-terminal region (Ogawa and Oshima, 1990), and binds at a specific UAS sequences in PHO promoters (Ogawa et al., 1994). The N-terminal region (amino acids 1 to 109), which is rich in acidic amino acids, was suggested to act as the activation domain for gene transcription and a region between amino acids 203 to 227 might be involved in oligomer formation.

The nucleotide sequence of PHO2 predicts that Pho2p is composed of 519 (Yoshida et al., 1989a) or 559 (Sengstag and Hinne, 1987) amino acid residues, and contains a homeodomain sequence between amino acids 76 to 135 or between amino acids 77 to 136 (Bürglin, 1988). A portion of the homeodomain sequence of Pho2p has a similar amino acid sequence to that of a short stretch found in Pho4p. Pho2p also has a stretch of glutamine residues (14 of 18 amino acids) on the N-terminal side of the homeodomain sequence. It was shown that Pho2p forms a ternary complex with Pho4p on a PHO promoter (Barbaric et al., 1996) by binding at A/T-rich segment(s) beside the UAS site (Magbanua et al., 1997a). It is thought that the Pho2p/Pho4p/UAS ternary complex may interact with the TBP, TFIIA, and TFIIEβ, and initiate transcription of the PHO genes (Magbanua et al., 1997b).

Proteins involved in the transduction of P, signals to Pho4p. Genetic evidence indicated that the negative regulators, Pho80p and Pho85p, inhibit Pho4p function but not the expression of PHO4 or PHO2 (Oshima, 1982; Yoshida et al., 1989a). Pho80p, deduced from the nucleotide sequence of PHO80, is a 34-kDa protein (Uesono et al., 1992) composed of 293 amino acid residues (Toh-e and Shimauchi, 1986). A coordinated increase of PHO80 dosage was found to cancel the dosage effect of PHO4 on the expression of PHO5 (Yoshida et al., 1989b) and a mutation occurring in PHO80 suppressed a specific PHO4 mutation (Okada and Toh-e, 1992). Based on these findings, it was thought that Pho80p directly interacts with Pho4p for the transduction of P, signals. On the other hand, Pho85p, encoded by PHO85 which contains an intron at the 6th codon, is a 36-kDa 305-amino acid protein known as a homologue of Cdc28p, a cyclin-dependent protein kinase of S. cerevisiae (Toh-e et al., 1988; Uesono et al., 1987, 1992). It was thought that Pho85p kinase phosphorylates Pho80p, resulting in Pho80p activation (Uesono et al., 1992). Recent re-examination of the amino acid sequence of Pho80p, however, has revealed that it has significant similarities to those of two yeast cyclins, Hsc26p (Ogas et al., 1991) and Orf5 (Fröhlich et al., 1991) in a conserved region of the cyclins (Kaffman et al., 1994). Pho80p and Pho85p form a complex and this complex, but neither protein alone, efficiently phosphorylates Pho4p in vitro and in vivo (Kaffman et al., 1994).

It was believed that the role of the cyclins and cyclin-dependent kinases is in cell cycle control. Pho80p and Pho85p, however, are thought to be unrelated to the cell cycle. This suggests a broader biochemical repertoire for cyclins and cyclin-dependent kinases in yeast (O’Neill and O’Shea, 1995). Pho85p kinase activity is apparently not restricted to the PHO system. A cyclin determines the specificity of the partner cyclin-dependent kinase, and Pho85p seems to associate with two other cyclins, Hsc26p and
OrfDp, besides Pho80p (Lenburg and O’Shea, 1996). Since both of these cyclins have been implicated in cell cycle regulation, Pho85p might therefore link cell-cycle events to the cell’s nutritional need for phosphate.

According to the genetic analysis, the pho81 mutation is hypostatic under the pho80 and/or pho85 mutation (Oshima, 1982). This fact indicates that Pho81p functions in the upstream of the Pho80p/Pho85p complex (Fig. 1). The nucleotide sequence of PHO81 suggests that Pho81p is a 134-kDa 1179-amino acid protein which has six repeats of a 33-amino acid sequence homologous to the ankyrin repeat (Lux et al., 1990) between amino acids 424 to 656 and a 33-amino acid sequence homologous to the ankyrin repeat (Lux et al., 1990) between amino acids 222 to 254 (Ogawa et al., 1993). The transcription of PHO81 is regulated by P, via the PHO regulatory system including both Pho4p and Pho2p (Fig. 1). Experiments with a chimeric gene consisting of the PHO81 ORF connected with the GAL1 promoter revealed that stimulation of the PHO regulatory system requires both increased transcription of PHO81 and P,-starvation conditions (Ogawa et al., 1995a). These findings suggest that Pho81p is inactive under high-P, conditions.

In a low-P, medium, Pho81p binds with and inhibits the kinase activity of the Pho80p/Pho85p complex (Ogawa et al., 1995a; Schneider et al., 1994), and allows underphosphorylated Pho4p to be imported into the nucleus and to turn on the transcription of PHO genes (Fig. 2). In the high-P, condition, Pho81p is, however, inactive as an inhibitor. Thus, the Pho80p/Pho85p complex is active for hyperphosphorylation of Pho4p and turns off the transcription of PHO genes, as described above. Hirst et al. (1994), however, claimed that Pho81p interacts directly with Pho4p, along with Pho80p, under conditions of PHO repression and that Pho80p and Pho81p dissociate from Pho4p under derepressive conditions.

The minimum amino acid sequence required for the above Pho81p function in vivo was narrowed down to a 141-amino acid segment (amino acids 584 to 724), which contains the fifth and sixth repeats of the ankyrin-like motif (Ogawa et al., 1995a; Schneider et al., 1994), and allows underphosphorylated Pho4p to be imported into the nucleus and to turn on the transcription of PHO genes (Fig. 2). In the high-P, condition, Pho81p is, however, inactive as an inhibitor. Thus, the Pho80p/Pho85p complex is active for hyperphosphorylation of Pho4p and turns off the transcription of PHO genes, as described above. Hirst et al. (1994), however, claimed that Pho81p interacts directly with Pho4p, along with Pho80p, under conditions of PHO repression and that Pho80p and Pho81p dissociate from Pho4p under derepressive conditions.

The minimum amino acid sequence required for the above Pho81p function in vivo was narrowed down to a 141-amino acid segment (amino acids 584 to 724), which contains the fifth and sixth repeats of the ankyrin-like motif (Ogawa et al., 1995a; Schneider et al., 1994), and allows underphosphorylated Pho4p to be imported into the nucleus and to turn on the transcription of PHO genes (Fig. 2). In the high-P, condition, Pho81p is, however, inactive as an inhibitor. Thus, the Pho80p/Pho85p complex is active for hyperphosphorylation of Pho4p and turns off the transcription of PHO genes, as described above. Hirst et al. (1994), however, claimed that Pho81p interacts directly with Pho4p, along with Pho80p, under conditions of PHO repression and that Pho80p and Pho81p dissociate from Pho4p under derepressive conditions.

The third to sixth repeats of the ankyrin-like motif of Pho81p have significant similarities to that of p16INK4 which inhibits the activity of the human cyclin D/Cdk4 kinase complex (Serrano et al., 1993). Deletion analyses revealed that the N- and C-terminal regions of Pho81p function as negative and positive regulatory domains, respectively, for the minimal 141-amino acid region (Ogawa et al., 1995a). The negative regulatory activity of the N-terminal domain was antagonized by a C-terminal segment of Pho81p supplied in trans. All four known classes of PHO81 mutations that conferred significant rAPase activity on cells grown in a high-P, medium occur in the N-terminal half of Pho81p.

### Specific UAS sequence for Pho4p binding

Genetic evidence suggests that Pho2p and Pho4p bind to or interact with a specific UAS in the promoter region of the PHO structural genes (Oshima, 1982). Such sites of PHO5 have been studied in several laboratories (Bergman et al., 1986; Nakao et al., 1986; Rudolph and Hinnen, 1987). The proposed nucleotide sequences for the UAS function by these authors were, however, in conflict with each other. To clarify the Pho4p-binding site, we investigated the PHO8 promoter, because only Pho4p, and not Pho2p, is required for PHO8 transcription. We found that a 6-bp sequence, CACGTG, at nucleotide position −535 relative to the ATG start codon (Fig. 3) is the Pho4p binding site in the PHO8 promoter, as determined by deletion, modification, and gel retardation analyses (Hayashi and Oshima, 1991). The protection of this CACGTG sequence by purified Pho4p from DNase I digestion in vitro was also shown (Barbarić et al., 1992; Ogawa et al., 1994).

Two putative UASs in the PHO5 promoter, designated UAS,1 in the region from nucleotide positions −373 to −347 and UAS,2 located from nucleotide positions −262 to −239 (Fig. 3), have been detected by in vitro binding of Pho4p (Vogel et al., 1989). These regions contain one copy each of, respectively, the CACGTG and CACGTG motifs (Fig. 4). We confirmed that purified Pho4p protects these sites...
from DNase I digestion in vitro (Ogawa et al., 1994). In
the same experiment, we found that another 21-bp region,
bearing a CATGTG sequence, in the PHO5 promoter from
nucleotide positions –202 to –182 (Figs. 3 and 4) was also
protected weakly by Pho4p. Deletion of the above 21-bp
region resulted in lower activity of PHO5 transcription (Rudolph and Hinnen, 1987). Thus, this 6-bp sequence,
designated as UASp3, might also be involved in the
PHO5 regulation.

We also examined UASs in the PHO81 promoter (Ogawa
et al., 1993). Results of a deletion analysis showed that a
95-bp region, extending from nucleotide positions –385 to
–291, could sense the Pi signals. A 19-bp region in the 95-
bp fragment was protected by Pho4p from DNase I diges-
tion in vitro (Fig. 4). We found one copy of the CACGTG
motif in the 19-bp region and no other CACGTG or CACGTT
motifs in the 95-bp region. It is known that PHO81 tran-
scription is severely reduced in a pho2 mutant (Yoshida et
al., 1989b). The above 95-bp fragment, however, could not
respond to a pho2 disruption allele, but a 488-bp fragment,
bearing a region of PHO81 DNA from nucleotide positions
–385 to +103, could respond to the pho2 disruption on a
YCp-type plasmid (Ogawa et al., 1993). This suggests that
a specific site for interaction with Pho2p might be located
in a region downstream of nucleotide position –290. A
CAAGTT sequence was found at nucleotide position –233
in the PHO81 promoter (Fig. 3), but the function of this
sequence remains to be examined.

The promoter region of PHO84 bears five copies of the 6-
bp motifs at nucleotide positions –880 (site A), –587 (site
B), –436 (site C), –414 (site D), and –262 (site E) relative to
the ATG start codon (Fig. 3). Binding of Pho4p to these
five copies of the 6-bp motif was confirmed by DNase I
footprinting (Ogawa et al., 1994). We found the highest
affinity for Pho4p at site D, whereas sites C and E had some-
what weaker affinities than site D. The A and B sites also
had some affinity but much weaker than site E. Analyses
of the promoter with deletion and base substitutions in the
above 6-bp motifs revealed that the 6-bp motifs at site C or
D and E are essential and sufficient for the full regulation
of PHO84 transcription, whereas sites A and B are dispens-
able (Ogawa et al., 1995b).

Eight effective UAS sites, each one located in the middle
of the 19 to 22 bp footprinting regions, protected by Pho4p
from DNase I digestion in vitro, in the promoters of PHO5,
PHO8, PHO81, and PHO84 (Ogawa et al., 1994), were com-
pared along with a sequence found at the distal site in the
PHO8 promoter (Barbarić et al., 1992). We found that six
of the UASs have the CACGTG sequence (type 1; Fig. 4)
and three have the CACGTT sequence (type 2).

The 6-bp CACGTG core sequence has been found to have
similarity to a highly conserved octanucleotide, RTCACRTG
(where R is a purine residue) called CDEI, located in cen-
tromeres and in the promoters of various genes for methio-
nine synthesis in S. cerevisiae (Baker and Masison, 1990;
Cai and Davis, 1990; Mellor et al., 1990). The CDEI-bind-
ing protein (i.e., Cpf1p, CP1 or Cbf1p) has a bHLH struc-
ture in its DNA-binding domain. Although crosstalk was
not detected between the phosphatase and methionine
genes in wild-type cells, overexpression of PHO4 was found

---

**Fig. 3.** Distribution of the 6-bp motif, CACGTK (K = G or T), in the promoter regions of the PHO genes. The open box at the right end of individual genes represents an ORF. Short open and hatched boxes indicate CACGTG and CACGTT motifs, respectively. Three 6-bp sequences, analogous to the 6-bp motif, shown in the PHO5, PHO8, and PHO81 promoters are indicated with their sequences (lowercase letters indicate deviation from the 6-bp motifs). The region in the PHO84 promoter shown with a broken line bearing sites A and B is dispensable for regulatory function. The function and Pho4p binding ability of the sequence analogous to the 6-bp motif in the PHO8 promoter (marked with an asterisk) were described by Barbarić et al. (1992), but that of the PHO81 promoter has not yet been examined.

---
The phosphatase system in *Saccharomyces cerevisiae* to suppress the methionine auxotrophy of a *cep1 (= cpf1)* mutant (O'Connell and Baker, 1992), under conditions of methionine starvation (O'Connell et al., 1995). In contrast, *CEP1* overexpression failed to suppress the rAPase – phe-notype of a *pho4* mutant (O'Connell and Baker, 1992). In this context, we speculated that the 6-bp sequence, CACGTG, is not sufficient for the specific recognition of a cognate positive regulator, and that the nucleotide sequences flanking the 6-bp sequence might have important functions in the discrimination of Pho4p and Cpf1p. This view was supported by the findings that flanking by a T residue at the 5'-end of the CACGTG motif is preferable for Cpf1p interaction but not for Pho4p interaction (Fisher and Goding, 1992). We found that the 6-bp motif regulated by Pho4p was flanked by a G residue at the 5' end and GG (for type 1 core sequence) or TT (for type 2 core sequence) residues at the 3' end (Fig. 4). Therefore, we proposed that the GCACGTGGG and GCACGTTTT motifs are consensus sequences in the *PHO* promoter (Ogawa et al., 1995b). Recent analysis of a complex of the bHLH domain of Pho4p with UAS DNA by X-ray crystallography supported the significance of the G and GG residues flanking, respectively, at 5'- and 3'-ends of the CACGTG motif (Shimizu et al., 1997). The observation that *CEP1* overexpression could not suppress a *pho4* mutation (O'Connell and Baker, 1992) suggests that Cpf1p has no affinity to the GCACGTTTT sequence of UAS1 in the nucleosome-free region (Almer and Hörz, 1986) of the *PHO5* promoter.

**Pho2p/Pho4p binding is aided by A/T-rich segment(s).** The transcription of all these *PHO* structural genes, except *PHO8*, is dependent on the other positive regulator, Pho2p. Thus, Pho2p might also be involved in the *PHO* regulation. It was demonstrated by use of the two-hybrid system that Pho2p interacts with Pho4p depending on the Pi concentration in the cultivation medium (Hirst et al., 1994) and the accessibility of the activation domain of Pho4p to the transcriptional machinery is enhanced by the interaction with Pho2p (Shao et al., 1996). Barbarić et al. (1996) reported that Pho4p and Pho2p form a ternary complex with UAS DNA and that a possible Pho2p binding site partially overlaps the UASp1 site with a GCACGTTTT motif. We could also detect direct interaction between Pho4p and Pho2p using immunoprecipitation and a protein binding assay (Magbanua et al., 1997b). This interaction results in an increased binding affinity of Pho4p to the UAS. Both the Pho4p and Pho2p proteins bind or interact with the basal transcription factors TBP, TFIIA, and TFIIIEβ. These observations suggest that the *PHO* gene transcription is initiated by direct contact of the Pho4p/Pho2p complex with the transcription machinery.

In the evaluation of the above 9-bp motifs on their transcriptional activity, we observed that a short A/T-rich segment(s) flanking the 9-bp motif has a role in the binding of Pho2p (Magbanua et al., 1997a), as suggested by Parent et al. (1994). Various 30-bp fragments of different *PHO* promoters, each bearing a 9-bp motif in the middle, and connected with 6-bp Xhol cohesive ends, were inserted

<table>
<thead>
<tr>
<th>Site</th>
<th>Pho4p binding sequence</th>
<th>Affinity to Pho4p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PHO5</em> UAsp2</td>
<td>-259 C A C A C G T G G A C T A G C -400</td>
<td>High</td>
</tr>
<tr>
<td><em>PHO8</em> Site D</td>
<td>-421 C T C C A G A C C A G T G G A C G G A -602</td>
<td>High</td>
</tr>
<tr>
<td><em>PHO8</em> UAS</td>
<td>-350 T A T G G C A C G T G G A A A A -332</td>
<td>High</td>
</tr>
<tr>
<td><em>PHO8</em> Proximal</td>
<td>-520 G T G A T C G C T G A C C G T G G A C G A -541</td>
<td>High</td>
</tr>
<tr>
<td><em>PHO5</em> UAsp3</td>
<td>-182 T A T T T G C A A C G T G G A A C T A T C C -202</td>
<td>Low</td>
</tr>
<tr>
<td><em>PHO8</em> Site C</td>
<td>-476 A C G T C C A C G T G G A A C T A T T -444</td>
<td>Low</td>
</tr>
<tr>
<td>Consensus 1</td>
<td>t t - - - G C A C G T G G G - c - a</td>
<td></td>
</tr>
<tr>
<td><strong>Type 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PHO5</em> UAsp1</td>
<td>-210 T A A A T T A C A C G T G T T T G C -352</td>
<td>Medium</td>
</tr>
<tr>
<td><em>PHO84</em> Site E</td>
<td>-251 A A A T A C G A C G T G T T T A A T C T A -271</td>
<td>Medium</td>
</tr>
<tr>
<td><em>PHO8</em> Distal</td>
<td>-718 T T A A A A A G C A C G T G T T T A A A A -737</td>
<td>Low</td>
</tr>
<tr>
<td>Consensus 2</td>
<td>a a t - - - G C A C G T T T T</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Regions in the *PHO* promoters protected from DNase I digestion in vitro by Pho4p. The protected sequences observed in previous studies (Ogawa et al., 1993, 1994) are listed for strands having sequences conforming most closely to GCACGTTGGG or GCACGTTTT along with the data of Barbarić et al. (1992) for the similar sequences at the distal site in the *PHO8* promoter. These sequences were classified into two groups depending on whether they contained the CACGTG (type 1) or CACGTT (type 2) sequence in the core region. The relative affinity of each sequence to Pho4p was estimated from the amount of Pho4p protein required for protection from DNase I digestion (Ogawa et al., 1994). The numerals at both ends of the sequences indicate nucleotide position of the 5'- and 3'-ends of the sequences in the promoter relative to the translation initiation codon of the respective genes.
into a CYC1-lacZ fusion gene on a YCp- or YEpl-type plasmid. Then the β-galactosidase activities of the yeast cells with the wild-type or pho2 disruptive allele and harboring one of the above plasmids were examined. Although the CYC1 promoter was reported to respond to a pho2 mutation (Sengstag and Hinnen, 1988), we confirmed that the CYC1 promoter fragment used was unresponsive to Pho2p and P, signals. No expression of the lacZ gene was detected with the above 36-bp fragment bearing UASp2 of PHO5 which possessed the ACACGTGGG sequence (Magbanua et al., 1997a). Similar 36-bp fragments each bearing UASp1 (GCACGTTTT) of PHO5, site D (GCACGTTTT) or E (GCACGTTTT) of PHO84 showed UAS activity in response to P, concentration in the medium in the wild-type cells, but not in the pho2 disruptant cells. We found that the Pho2p-responsive UASs are flanked by one or two copies of an A/T-rich segment, whereas UASp2 is not (Fig. 5). Gel retardation and competition experiments with Pho2p, tagged with T7 epitope on N-terminal and six histidine residues at C-terminal, showed that the chimeric protein binds to the 36-bp fragments bearing A/T-rich segment(s) but not appreciably to that without A/T-rich segment(s). These facts strongly suggest that the A/T-rich segments flanking the PHO UASs are Pho2p binding sites and play an important role in PHO regulation.

Pho2p also binds to the HIS4, ADE2, ADE5,7, and TRP4 promoters. The Bas1p and Bas2p (= Pho2p) proteins are both required for activation of basal transcription of the HIS4 gene. The results of DNase I footprinting experiments (Tice-Baldwin et al., 1989) demonstrated that Pho2p binding site, which is rich in A/T pairs, partially overlaps the Bas1p binding site (Fig. 5). It was known that the core TGACTC sequence alone, to which Bas1p binds, is not sufficient for the proper regulation of the ADE2 and ADE5,7 promoters unless a Pho2p binding site is also present (Daignan-Fornier and Fink, 1992). The Bas1p binding sites, shown by the above authors in the ADE2 and ADE5,7 promoters, are flanked by one or two copies of A/T-rich segment(s) (Fig. 5). Although the Pho2p binding of the ADE1 and ADE8 promoters has not been demonstrated.

---

**Fig. 5. Alignment of UAS sequences to which relevant regulatory proteins bind together with Pho2p.** The consensus sequences in the promoter for Bas1p (Daignan-Fornier and Fink, 1992), Pho4p (Barbaric et al., 1996; Ogawa et al., 1994, 1995b), and Swi5p (Brazas and Stillman, 1993b) were as described. The core UAS sequences for binding of the indicated proteins are written in boldface letters and the regions protected in the footprinting experiments are indicated by brackets. The asterisks indicate that the Bas1p binding sites in the ADE1 and ADE8 promoters were identified by sequence comparison (Daignan-Fornier and Fink, 1992), Pho2p binding sites identified in footprinting experiments are indicated by double underlines, A/T-rich segments are singly underlined. The broken underline indicates the palindromic sequence flanking the 9-bp UAS motif of PHOS. The numerals at both ends of the sequences indicate nucleotide position of the 5'- and 3'-ends of the sequences in the promoter of the respective genes.
in vitro, the alignment of their promoter sequences indicates the presence of A/T-rich segment(s) flanking the TGACTC motif (Fig. 5). Pho2p also binds to the promoter of the tryptophan biosynthetic TRP4 gene at the A/T-rich UAS, site, to which the general control regulator, Gcn4p, binds (Braus et al., 1989). In this case, Pho2p and Gcn4p, however, bind to the UAS, site in a mutually exclusive manner. Pho2p recognizes similar A/T-rich segments at a region adjacent to the Swi5p binding site in the HO promoter (Brazas and Stillman, 1993a,b). This was also supported by the experiments with increased spacing between putative Pho2p and Swi5p binding sites with A/T pair of nucleotides in the HO promoter (Brazas et al., 1995).

The 36-bp PHO81 UAS fragment was found to have 4- or 5-bp A/T segments flanking the 9-bp UAS motif had weak affinity to Pho2p and could not exhibit appreciable UAS activity under the normal dosage of Pho4p (Magbanua et al., 1997a). We speculated that the 4- and 5-bp A/T segments at the PHO81 UAS are covered completely by Pho4p as it protects a 19-bp region, bearing these A/T segments, from the DNase I digestion (see Fig. 5). Thus, it may result in a reduced level of UAS activity due to the ineffective binding of Pho4p and/or Pho2p to the PHO81 UAS. The GCAAGTTTT sequence at nucleotide position –234 in the region adjacent to the Swi5p binding site in the PHO81 promoter (Ogawa et al., 1993) is flanked with a long A/T-rich segment at the 5′ side, but not on the 3′ side. In contrast, the similar 36-bp PHO8 UAS fragment has no apparent A/T-rich segment and no appreciable binding affinity to Pho2p was observed. However, the level of expression of the CYC1-lacZ fusion gene inserted with the 36-bp PHO8 UAS fragment under high-P, conditions was relatively high and the differential ratio of the lacZ expression in low- and high-P, media was about 2.5 (Magbanua et al., 1997a) as in the case of the genomic PHO8 expression (Kaneko et al., 1987; Toh-e et al., 1976). This may be due to the fact that the 9-bp UAS of PHO8 has a 10-bp palindromic sequence, AGTGAATCGCT, adjacent to it (Fig. 5). This sequence may respond to another unknown regulatory protein.

CONCLUDING REMARKS

We can now attain substantial understanding into the broad outline of function of the regulatory genes and proteins in the PHO system. However, many questions remain unanswered. One of the major unsolved mechanisms is of how extracellular P, signals are detected. In the recent genetic study of mutants exhibiting a defect in P, uptake, we could identify three putative membrane proteins, Pho86p, Pho87p, and Pho88p, which might be involved in the P, uptake mechanism in association with Pho84p and Gtr1p (Bun-ya et al., 1996; Yompakdee et al., 1996a,b). Among them, Pho86p and Pho88p are particularly interesting as they were suggested to modulate Pho81p function in accordance with extracellular P, concentration.

Another question remaining to be addressed is “what is the consequence of the Pho4p/Pho2p/UAS ternary complex formation in recruiting the transcriptional machinery to the PHO genes?”. It was argued that nucleosome remodeling is evidently a direct consequence of the recruitment of the transcriptional machinery (Ptashne and Gann, 1997). Hořz and his colleagues have examined this idea using the PHO5 gene (see review, Svaren and Hořz, 1997). These authors proposed that the PHO5 promoter is protected by positioned nucleosomes in repressed conditions, except for the UAS, 1 site which is positioned at a nucleosome-free site. Upon derepression of PHO5, disruption of nucleosomes occurs due to the binding of Pho4p at the UAS, 1 site, possibly in association with Pho2p. Then the binding of an additional Pho4p molecule at another site, UAS, 2, induces removal of four nucleosomes from the promoter, thereby facilitating the binding of the transcriptional machinery to the promoter. Thus, these authors claimed that the chromatin disruption at the promoter is the prerequisite for transcription of the PHO5 gene. The powerful combination of genetic and biochemical studies available in S. cerevisiae will lead to a more detailed and comprehensive understanding of the above questions in the near future.

Our studies cited in this article were carried out in the Department of Biotechnology (formerly Department of Fermentation Technology), Osaka University in collaboration with A. Toh-e, S. Harashima, Y. Kaneko, K. Yoshida, N. Ogawa, N. Hayashi, M. Bunya, and many students, and were supported by grants for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

homeodomain protein Pho2 and the basic-helix-loop-helix protein Pho4 bind DNA cooperatively at the yeast PHO5 promoter.

Nucleic Acids Res. 24, 4479–4486.


Biochemical and genetic evidence that yeast extracellular protein phosphatase activity is due to acid phosphatase.


