MOLECULAR AND CELLULAR BIOLOGY, Feb. 1991, p. 785–794
0270-7306/91/020785-10$02.00/0

Vol. 11, No. 2

Specific *cis*-Acting Sequence for *PHO8* Expression Interacts with PHO4 Protein, a Positive Regulatory Factor, in *Saccharomyces cerevisiae*

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Received 5 July 1990/Accepted 8 November 1990

The *PHO8* gene of *Saccharomyces cerevisiae* encodes repressible alkaline phosphatase (rALPase; EC 3.1.3.1). The rALPase activity of the cells is two to three times higher in medium containing a low concentration of P_i than in high-P_i medium due to transcription of *PHO8*. The P_i signals are conveyed to *PHO8* by binding of PHO4 protein, a positive regulatory factor, to a promoter region of *PHO8* (*PHO8p*) under the influence of the *PHO* regulatory circuit. Deletion analysis of *PHO8p* DNA revealed two separate regulatory regions required for derepression of rALPase located at nucleotide positions −704 to −661 (distal region) and −548 to −502 (proximal region) and an inhibitory region located at −421 to −289 relative to the translation initiation codon. Gel retardation experiments showed that a β-galactosidase–PHO4 fusion protein binds to a 132-bp *PHO8p* fragment bearing the proximal region but not to a 226-bp *PHO8* DNA bearing the distal region. The fusion protein also binds to a synthetic oligonucleotide having the same 12-bp nucleotide sequence as the *PHO8p* DNA from positions −536 to −525. The 132-bp *PHO8p* fragment, connected at position −281 of the 5′ upstream region of a *HIS5′-′lacZ* fused gene, could sense P_i signals in vivo, but a 20-bp synthetic oligonucleotide having the same sequence from −544 to −525 of the *PHO8p* DNA could not. Linker insertions in the *PHO8p* DNA indicated that the 5-bp sequence 5′-CACGT-3′ from positions −535 to −531 is essential for binding the β-galactosidase–PHO4 fusion protein and for derepression of rALPase.

Cells of *Saccharomyces cerevisiae* have a nonspecific repressible alkaline phosphatase (rALPase; EC 3.1.3.1) in vacuoles (7). The *PHO8* gene on chromosome IV encodes this enzyme (16), and its expression is regulated at a transcriptional level by the P_i concentration in the medium (14); rALPase activity is three times higher in low-P_i medium than in high-P_i medium. The P_i concentration in the cultivation medium is registered through a system consisting of the products of the *PHO4*, *PHO80*, *PHO81*, and *PHO85* genes (25, 40, 42). The function of another gene, *PHO9* (*PEP4* [12, 16, 43]), encoding proteinase A (2, 38, 43), is also indispensable for processing the PHO8 polypeptide.

The function of PHO4 protein, a positive regulatory factor, is indispensable for derepression of *PHO8* transcription (14), while the *PHO80* product negatively controls PHO4 function under the influence of P_i through the product of the *PHO81* gene. The PHO4 and PHO80 proteins are suggested to interact with each other directly (42). The *PHO85* gene encodes a protein having an amino acid sequence homologous with those of protein kinases and is thought to act as an activator of the PHO80 protein (35, 36).

The same regulatory system as for *PHO8* expression, in combination with an additional positive regulatory factor encoded by the *GRF10* gene (formerly named *PHO2* [42] or *BAS2* [3]), coordinately controls the transcription of *PHO5*, encoding repressible acid phosphatase (rAPase; EC 3.1.3.2 [17]), *PHO84* for a P_i transport system (31), and *PHO81*, one of the regulatory genes for the phosphatase regulon (40, 42). However, no direct function of *GRF10* is necessary for *PHO8* derepression (31, 34). The positive regulatory factors PHO4 and GRF10 are both thought to interact with a specific upstream activating sequence (UAS) of these structural genes for their transcriptional regulation. Nakao et al. (22) and Bergman et al. (4) have proposed the existence of two copies of a 10-bp sequence directly repeated in the promoter region of *PHO5* (*PHO5p*). On the other hand, Rudolph and Hinnen (27) have suggested that three copies of specific 19-bp dyad sequences, different sequences from those suggested by Nakao et al. (22) and Bergman et al. (4), fulfill the function of transcriptional control of *PHO5*. The possibility that the PHO4 protein binds to *PHO5p* is supported by its retardation of the mobility of *PHO5* DNA on gel electrophoresis (24, 37). Vogel et al. (37) reported that two of the three 19-bp dyad sequences suggested by Rudolph and Hinnen (27), UASp1 (starting at position −376) and UASp2 (at −254), bind to PHO4 protein, but that these two sequences have little homology with each other except for a pentamer, 5′-CACGT-3′.

GRF10 protein is also directly related to the amount of *PHO5* and *PHO81* mRNAs (40, 42), suggesting that it also binds to the promoter sequences of these genes. A modified *PHO5p* DNA with a deletion of a putative binding site for GRF10 protein, however, has normal function for *PHO5* expression (27, 37). In addition, a 31-bp sequence from positions −381 to −351, which contains UASp1 but not the putative GRF10 binding site, could confer the ability to sense P_i signals for the promoter DNA of *CYC1* (29), where the 31-bp sequence was inserted, in the presence of the *PHO4* and *GRF10* genes. Another binding site for the GRF10 protein was suggested to be located in a sequence slightly overlapping UASp2 (37). Therefore, it is possible that the sequences proposed by the above authors are sites of interaction with a complex of the PHO4 and GRF10 proteins. To clarify these ambiguities and to identify the PHO4 protein binding sequence, we investigated the specific

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cis-acting regulatory sequence in the upstream region of *PHO8*, because only the PHO4 protein, without the GRF10 protein, is involved in the transcriptional regulation of *PHO8* (25, 31, 34).

This communication reports two *cis*-acting regulatory regions and an inhibitory region of *PHO8p* revealed by deletion analysis. One of the regulatory regions, the proximal one relative to the translation initiation codon, could bind with a β-galactosidase–PHO4 fusion protein and was found to bear the specific 5-bp sequence 5′-CACGT-3′, suggested to be the PHO4 binding site in *PHO5* DNA by Vogel et al. (37). This 5-bp motif is essential for the binding of the PHO4 protein in vitro and for derepression of *PHO8* in vivo. The 6-bp sequence 5′-CACGTG-3′ was shown to be preferred to the 5-bp motif for *PHO8* expression. A 5′-CCACGTGCAGCG-3′ sequence was sufficient for binding of the PHO4 protein in vitro, whereas a 20-bp sequence including this 12-bp sequence was not sufficient for UAS function in vivo. A 132-bp fragment bearing the 47-bp proximal region but not the 44-bp distal region was sufficient to sense the P_i signals in vivo.

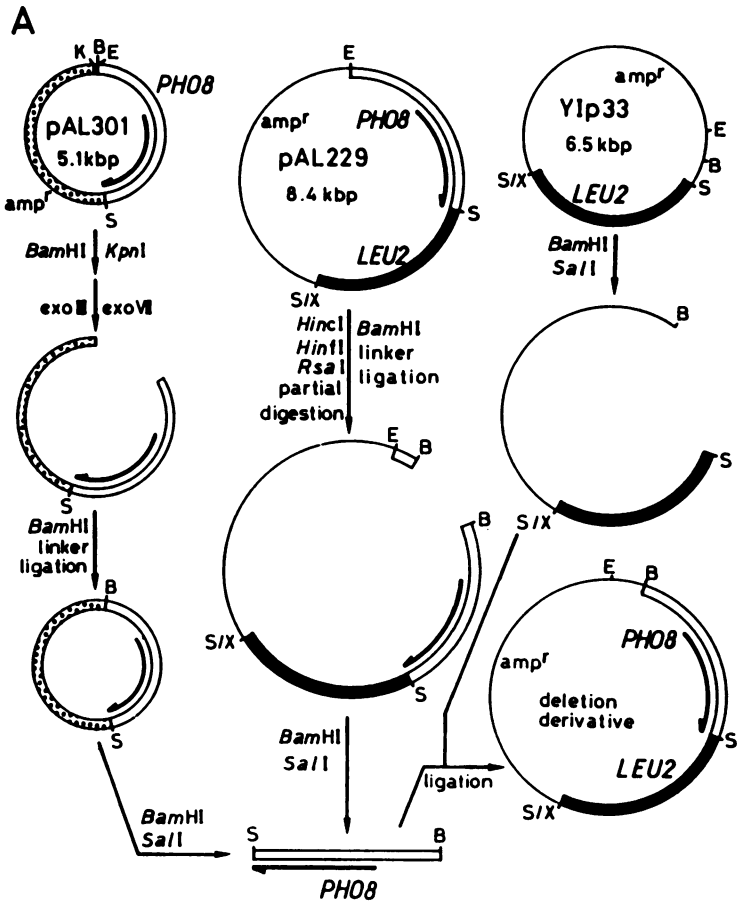
MATERIALS AND METHODS

Organisms, plasmids, and DNA fragments. *S. cerevisiae* K143-1C (*MATα pho3-1 pho13::pPH13 [ura3-1,2] leu2-3,112 lys1*) was used for determination of the phenotype conferred by a cloned *PHO8* fragment. The *PHO13* locus, encoding the specific *p*-nitrophenylphosphatase (*p*-NPPase [15]) of this strain was disrupted by insertion of plasmid pPH13 bearing *PHO13* DNA with deletion of the N- and C-terminal coding regions (13). Another yeast strain, K3-5D (*MATa pho3-1 leu2-3,112* [41]), was used as a host for determination of β-galactosidase activity conferred by a plasmid bearing the *HIS5′-′lacZ* fusion gene (23). *Escherichia coli* JA221 (8) was used for propagation of plasmid DNAs. Another *E. coli* strain, JM103 (19), was used for gene cloning with pUC19 (39) as the vector, preparation of single-stranded DNA with M13 bacteriophage for nucleotide sequencing (19), and propagation of a crude cell extract containing the β-galactosidase–PHO4 fusion protein. The constructions and structures of the principal plasmids used to manipulate the *PHO8* DNA are shown in Fig. 1. The other plasmids used were pUC19, pBR322 (30), YIp33 (26), YEp13 (26), and pUR290 and pUR291 (28).

For investigation of the *PHO8p* DNA, a 2.4-kbp *Eco*RI-*Sal*I fragment bearing the 989-bp 5′ upstream region and the 1,459-bp 5′ half of the *PHO8* open reading frame cloned on plasmid pAL201 (13, 14) was used. Since the wild-type *PHO8* gene has a 1,698-bp open reading frame, this 2.4-kbp fragment produces truncated rALPase polypeptide (13).

Media and biochemical methods. High-P_i or low-P_i versions of nutrient and synthetic media were prepared as described previously (33). For *E. coli*, L broth as nutrient medium and M9 as minimal medium were prepared as described before (20) and appropriately supplemented with nutrients with or without 50 μg of sodium ampicillin per liter, but 10 μg of sodium ampicillin per liter was added to the media for strain JM103 transformed with pUR290 or pUR291. *S. cerevisiae* was transformed by the Li-acetate method (11), and *E. coli* was transformed by the method of Morrison (21). For deletion of a portion of the *PHO8p* DNA, exonuclease III (Takara Shuzo Co. Ltd., Kyoto, Japan) and exonuclease VII (Bethesda Research Laboratories Life

FIG. 1. Construction and structure of the principal plasmids used. (A) Construction of plasmids bearing the *PHO8p* DNA with various deletions. Plasmid pAL301, used to create sequential end deletions of the *PHO8p* DNA, was constructed from plasmid pAL201 (14). The pAL201 DNA was restricted with *Eco*RI, filled in, and ligated with an 8-bp *Bam*HI linker. A 2.4-kbp *Bam*HI-*Sal*I fragment bearing the *PHO8* DNA from nucleotide positions −989 to +1459 was prepared from this pAL201 DNA and inserted into the *Bam*HI-*Sal*I gap at the polycloning site of pUC19. The resultant plasmid, pAL301, was double restricted with both *Bam*HI and *Kpn*I at the polycloning site of pUC19 and digested with exonuclease III to various extents from the *Bam*HI end of the larger fragment (in which the *Kpn*I end was not digested with exonuclease III). The sequences protruding at the two ends were eliminated by digestion with exonuclease VII and filled in with Klenow fragment, ligated with 8-bp *Bam*HI linkers, and circularized. The ends of these digested fragments were determined directly (39) or by subcloning the *Bam*HI-*Sal*I fragments on M13 bacteriophage (19). Plasmid pAL229 was constructed by insertion of a 2.2-kbp *Xho*I-*Sal*I *LEU2* fragment of *S. cerevisiae* prepared from YEp13 into the *Sal*I site of pAL201. Plasmid pAL229 was partially digested with *Hin*cII, *Hin*fI, or *Rsa*I to obtain truncated *PHO8p* fragments. Then the blunt ends created by *Hin*cII or *Rsa*I restriction were directly ligated with an 8-bp *Bam*HI linker or after the *Hin*fI restriction ends were filled in. The *Bam*HI-*Sal*I fragments of the *PHO8* DNA with various deletions were prepared from the modified pAL301 DNAs and similar fragments from modified pAL229 having *Bam*HI linker insertions in the *PHO8p* region. These fragments from nucleotide positions −989 to +1459 of the *PHO8* DNA with and without deletions in the *PHO8p* region illustrated in Fig. 2 were inserted into the *Bam*HI-*Sal*I gap of YIp33 bearing the *LEU2* marker. (B) Structures of the other principal plasmids. Plasmids pAL364 and pAL411 were constructed by insertion of a 132-bp *Hin*cII fragment of *PHO8p* DNA from positions −615 to −484 and a 226-bp *Bam*HI-*Bgl*II fragment of the same DNA from positions −841 (the exonuclease III-digested end connected to an 8-bp *Bam*HI linker) to −616 (*Hpa*I restriction end connected to an 8-bp *Bgl*II linker) into the *Hin*cII or *Bam*HI site, respectively, in the polycloning site of pUC19. Plasmid pAL447 was constructed as follows. An 8-bp *Bam*HI linker was inserted at position −616 in the *PHO8p* DNA of pAL229 after partial digestion with *Hpa*I, and this plasmid DNA was restricted with *Bam*HI and *Sal*I. The resultant 2.1-kbp *Bam*HI-*Sal*I fragment was inserted into a *Bam*HI-*Sal*I gap of YIp33, and an 8-bp *Xho*I linker was inserted into the resultant chimeric plasmid after partial digestion with *Hin*cII at position −484 of the *PHO8p* DNA. Plasmid pAL458 was constructed by ligation of 8-bp *Xho*I linkers at both ends of a 4.2-kbp *Hpa*I fragment of the *PHO8*-pBR322-*LEU2* DNA of the chimeric plasmid with deletion of the upper region of *PHO8p* from −841 (see third row, Fig. 2A). One of the two *Hpa*I sites ligated with the *Xho*I linkers is at position −616 on *PHO8p*, and the other *Hpa*I site is at position −369 in *LEU2* DNA. The 4.2-kbp fragment was then circularized by ligation at the *Xho*I ends. Thus, plasmid pAL458 has the 226-bp sequence of *PHO8p* DNA from positions −841 to −616. Plasmids pAL459, pAL460, and pAL461 carry three synthetic oligonucleotides, oligo-12, oligo-16, and oligo-20, respectively, at the *Bam*HI-*Sal*I gap of pBR322. Plasmid pSN2 was constructed by an *Xho*I linker insertion at the *Hin*fI site (position −282) in a 745-bp *Hin*dIII (position −616)-*Ava*II (+118) fragment connected with an 8-bp *Bam*HI linker at both ends of the *HIS5p* DNA cloned at the *Bam*HI site in pBR322 (23). Plasmid pSN616ΔB, constructed in a previous study (23), was used to examine the promoter functions of various DNA constructs consisting of the *PHO8p*, *HIS5p*, and *lacZ* DNAs. The open, solid, hatched, cross-hatched, and stippled boxes in pSN616ΔB, the dotted boxes in pAL301, pAL364, and pAL411, and the thin line represent yeast *PHO8*, *LEU2*, *HIS5*, 2μm plasmid, *E. coli lacZYA*, pUC19, and pBR322 DNAs, respectively. The arrows along the *PHO8* and *HIS5* genes indicate approximate positions and reading directions of the open reading frames. Restriction sites: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; Hc, *Hin*cII; Hp, *Hpa*I; K, *Kpn*I; P, *Pst*I; Pm, *Pma*CI; S, *Sal*I; Sm, *Sma*I; and X, *Xho*I.


A
pAL301
5.1kbp
PHO8
ampr
BamHI
KpnI
exoIII
exoVII
BamHI
linker
ligation
pAL229
8.4 kbp
LEU2
HincII
HinfI
RsaI
partial
digestion
BamHI
linker
ligation
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6.5 kbp
LEU2
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deletion
derivative
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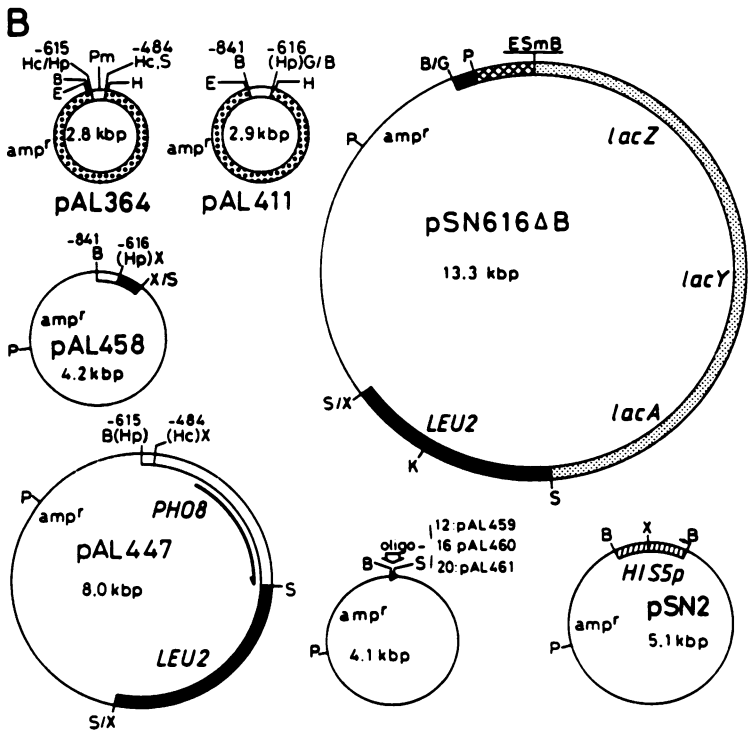
ligation
PHO8


B
pAL364
2.8 kbp
pAL411
2.9kbp
pAL458
4.2kbp
pAL447
8.0kbp
PHO8
LEU2
pSN616ΔB
13.3 kbp
lacZ
lacY
lacA
LEU2
ESmB
oligo-
12:pAL459
16 pAL460
20:pAL461
4.1 kbp
HIS5p
pSN2
5.1kbp

Technologies, Inc., Gaithersburg, Md.) were used in the conditions described by Yanisch-Perron et al. (39). Synthetic *Bam*HI, *Bgl*II, *Hpa*I, and *Xho*I linkers were purchased from Takara Shuzo, and synthetic *Bal*I linker was from Boehringer Mannheim GmbH, Biochemica (Mannheim, Federal Republic of Germany). The other general methods for modification and analysis of DNA have been described previously (23).

Synthetic oligodeoxynucleotides. Three pairs of complementary oligonucleotides were synthesized and annealed as described previously (10). The annealed DNA fragments were electrophoresed in a 15% polyacrylamide gel and eluted from the gel as described by Maxam and Gilbert (18). For the gel retardation assay, these and other restricted fragments were labeled at both protruding ends with [α-^{32}P]dATP, dCTP, dGTP, and dTTP, with use of the Klenow fragment of DNA polymerase I (Takara Shuzo).

Determination of enzyme activities. For assay of rALPase activities of *S. cerevisiae*, cell extracts were prepared from cells grown in 10 ml of the indicated medium. The cells were harvested, washed, and suspended in 0.2 ml of 25 mM Tris hydrochloride buffer (pH 8.5) containing 1 mM $MgSO_4$. The cells were disrupted by vigorous shaking in a Vortex mixer (six times, 30 s each) with 1 mg of glass beads (0.6 mm diameter, Toshinriko, Tokyo, Japan) at 4°C. Then, 0.2 ml more of the same buffer was added with mixing, and the glass beads were removed by low-speed centrifugation. The supernatant was centrifuged for 10 min at 12,000 rpm in an Eppendorf centrifuge. The rALPase activity of the final supernatant was determined as described by Toh-e et al. (34) with *p*-nitrophenylphosphate as the substrate. Detection and assay of β-galactosidase activity were performed as described previously (23).

Gel retardation assay. For the protein-binding study, PHO4 protein fused with β-galactosidase (β-Gal::PHO4) was prepared by expression of a DNA fragment named Z-4 bearing a *lacZ'-'PHO4* fusion gene on a pUR291-based plasmid in *E. coli* as described before (24). The fused gene produced β-Gal::PHO4 fusion protein consisting of a moiety of PHO4 protein from the fourth N-terminal amino acid to the C-terminus connected to the C-terminal amino acid of β-galactosidase with an RGSG spacer amino acid sequence originating from a 12-bp *Bam*HI linker used for the gene fusion. Thus, the β-Gal::PHO4 protein of Z-4 has the specific DNA-binding domain at the C-terminus of PHO4.

The indicated amount of ^{32}P-labeled *PHO8p* DNA was added in 40 μl of 25 mM Tris hydrochloride buffer (pH 7.5) containing 50 mM NaCl, 4% glycerol, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.05% Triton X-100, 0.1 mg of calf thymus DNA per ml, and 1 mM phenylmethylsulfonyl fluoride (binding buffer), and the mixture was supplemented with an appropriate amount of the β-Gal::PHO4 preparation and incubated for 40 min in an ice bath. Then the reaction mixture was supplemented with 20 μl of 50% glycerol solution, and a 15-μl portion of the mixture was charged into a slot in a polyacrylamide gel and electrophoresed in 6.7 mM Tris hydrochloride buffer (pH 7.9) containing 3.3 mM sodium acetate and 1 mM EDTA (low-ionic-strength buffer [6]).

RESULTS

Deletion analysis of *PHO8p* DNA. For determination of the essential regions for regulation, a series of deletion fragments of the *PHO8* DNA having sequential end deletions (Fig. 2A) were constructed as shown in Fig. 1A. Another series of four modified *PHO8p* fragments with internal deletions (Fig. 2B) were constructed by recombination of appropriate restriction fragments of the sequential end deletions and the wild-type *PHO8p* DNA. These *PHO8p* fragments with or without deletions were inserted into the *Bam*HI-*Sal*I gap of YIp33 bearing the *LEU2* marker. Three other deletion fragments (Fig. 2B, bottom) were constructed from the relevant fragments of the sequential end deletions truncated at positions −841, −660, and −628 by restriction at the *Hpa*I site (position −616). The fragments were connected with an 8-bp *Bgl*II linker at the *Hpa*I site, and the short *Bam*HI-*Bgl*II fragments obtained were inserted into the *Bam*HI site of the above YIp33-based chimeric plasmid bearing the *PHO8p* DNA truncated at position −501.

All the chimeric YIp33-based plasmids were restricted at the *Bgl*II site at position +570 in the *PHO8* open reading frame and used to transform *S. cerevisiae* K143-1C, which has a disrupted *pho13* locus to eliminate *p*-NPPase activity and the *leu2* mutation. We isolated several Leu$^+$ transformants. Chromosomal integration of the plasmid at the *PHO8* locus may result in two copies of the *PHO8* region, one copy consisting of a deleted promoter with an intact open reading frame and sufficient 3′ downstream region, and the other copy having an intact promoter with a truncated open reading frame. This was examined for all the Leu$^+$ transformants by restriction of the genomic DNAs with *Pst*I and Southern hybridization with a ^{32}P-labeled 1.1-kbp *Hin*dIII fragment of *PHO8* (from nucleotide positions −162 to +926) as a probe. When a single copy of the DNA construct was integrated, two hybridization bands of 11 and 4.8 kbp should be observed, whereas when two copies of the DNA construct were integrated, another hybridization band of 8 kbp should be detected in addition to the above two bands. All the integrants were confirmed to have a single-copy insertion of the DNA construct at the *PHO8* locus (data not shown).

The transformants were cultivated in YPD medium at 30°C for 2 days. Then the cells were washed with sterilized water and inoculated into 10 ml of the low-P_i or high-P_i version of the Leu test medium. The cultures were shaken at 30°C, and the rALPase activities of the cells in the late logarithmic phase were determined. The *PHO8* gene with a promoter truncated at nucleotide position −548 and those with longer promoters conferred substantial rALPase activity in low-P_i medium and showed about two- to five-fold higher activities in low-P_i medium than in high-P_i medium (Fig. 2A). However, the promoter truncated at position −501 showed significantly lower rALPase activity and no differential synthesis of rALPase in response to the P_i concentration. These facts indicate that a sequence critical for *PHO8* regulation is located in a 47-bp region between nucleotides −548 and −502 (proximal region; Fig. 3).

The *PHO8* fragments with deletions from nucleotides −616 to −483 and −583 to −365, both in the proximal region, still showed substantial regulatory function (Fig. 2B). A *PHO8p* fragment extending from position −841 to the ATG but with a deletion from positions −615 to −502 showed normal regulation, whereas another promoter fragment with the sequence from position −660 to the ATG but with a deletion from positions −615 to −502 did not respond to P_i signals. Another fragment with a deletion from −704 to −395 showed complete loss of regulatory function. These findings indicate the existence of another regulatory region in the 44-bp sequence between nucleotides −704 and −661 (distal region; Fig. 3).

Elimination of the upstream region from position −422 almost completely abolished the rALPase activity, whereas

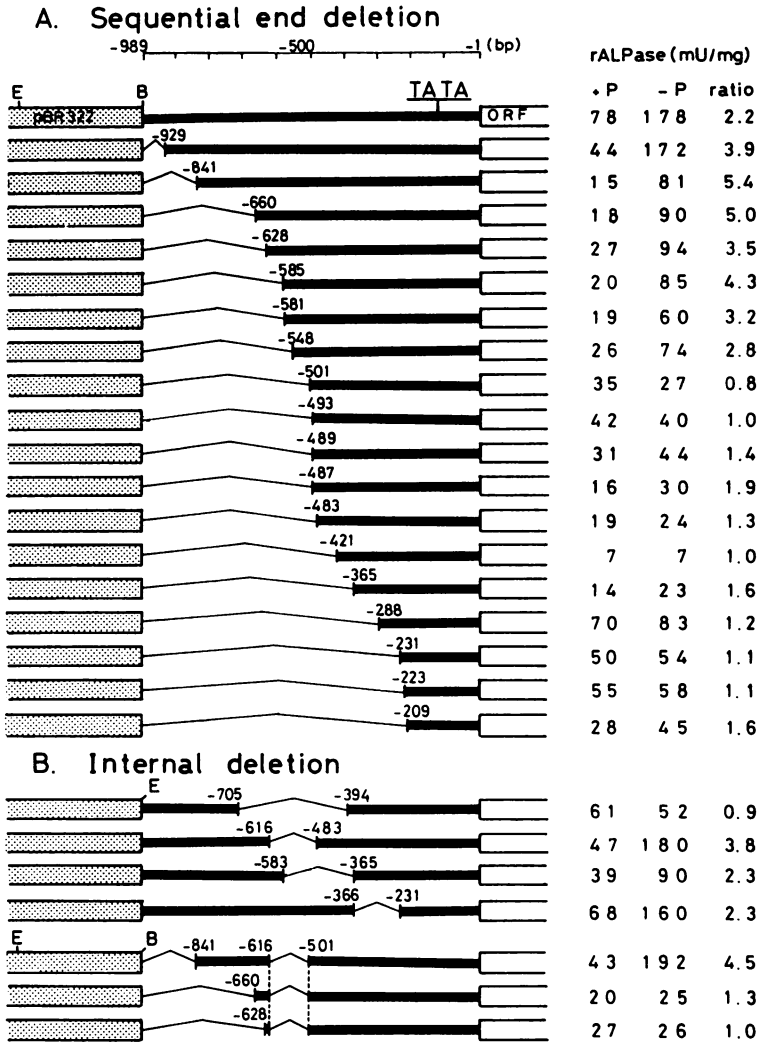


FIG. 2. Expression of *PHO8* DNAs with and without deletions in the promoter region. Transformants of *S. cerevisiae* K143-1C obtained by integration of single copies of chimeric YIp plasmids bearing the *PHO8* DNA were cultivated in YPD medium at 30°C for 2 days. The cells were then washed and inoculated into 10 ml of the low-P_i and high-P_i versions of the Leu test medium to give an initial OD_{660} of 0.1. The cultures were shaken at 30°C, and rALPase activities were determined when the cells reached the late logarithmic phase (OD_{660} of 1.0). Values for rALPase activity represent averages of values in at least three independent experiments on a number of different clones having the same integrated DNA construct. The standard deviations were less than 15% of the means. The dotted boxes represent pBR322, and the thick lines and open boxes represent the 5′ noncoding region and the open reading frame (ORF) of *PHO8* DNAs, respectively. Numbers above the bars indicate nucleotide positions relative to the translation initiation codon. Each end of the *PHO8p* DNA was ligated with an 8-bp *Bam*HI linker, except the three promoter fragments listed at the bottom of panel B, which had *Bgl*II linkers at nucleotide position −616. +P and −P indicate cultivation in high-P_i and low-P_i media, respectively. The values in the farthest right column are ratios of rALPase activities in low-P_i and high-P_i media. E, *Eco*RI; B, *Bam*HI.

elimination of all the upstream region from position −289 (Fig. 2A) and internal deletion of the region from −704 to −395 resulted in substantial enzyme activity but no response to P_i signals (Fig. 2B). These observations suggest that a 133-bp sequence from nucleotides −421 to −289 inhibits *PHO8* expression (inhibitory region; Fig. 3).

Nucleotide sequence for binding the β-Gal::PHO4 fused protein. We used the gel retardation assay to examine binding of the PHO4 protein with the above sequences in the *PHO8p* DNA. A 187-bp *Eco*RI-*Hin*dIII fragment, bearing a 132-bp *Hin*cII fragment of *PHO8p* from positions −615 to −484, involving the proximal region, was prepared from plasmid pAL364 (Fig. 1B). Both restriction ends of the

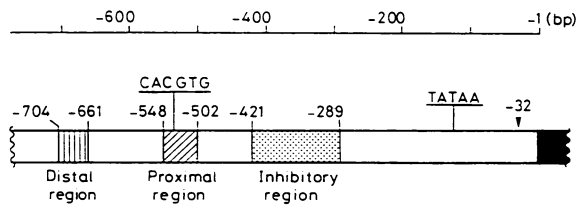


FIG. 3. Regulatory regions of *PHO8p* DNA. Numbers above the regions indicate nucleotide positions relative to the translation initiation codon of *PHO8*. The arrowhead at position −32 represents the major site for transcription initiation (13). The solid box indicates the open reading frame of *PHO8*.

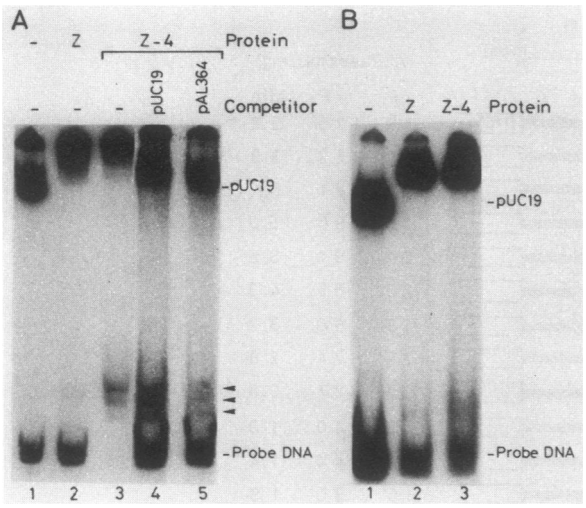


FIG. 4. Gel retardation assays of *PHO8p* DNA fragment. Plasmid DNAs of pAL364 (Fig. 1B) and pAL411 (Fig. 1B) were propagated in *E. coli*, double digested with *Eco*RI and *Hin*dIII, and filled in with [α-^{32}P]dATP, dGTP, dCTP, and dTTP. Samples (0.2 pmol) of the ^{32}P-labeled DNA fragments (A) from pAL364 bearing the proximal region of *PHO8p* DNA or (B) from pAL411 bearing the distal region of the *PHO8p* DNA were incubated with 10 μg of protein of the β-Gal::PHO4 preparation in 40 μl of binding mixture, except for lanes 1 of each panel, to which no protein was added. The binding mixtures were then incubated for 40 min in an ice bath. The proteins used for the binding reaction were crude extracts of *E. coli* harboring pUR290 (lane Z) and *E. coli* bearing the *lacZ'-'PHO4* fusion gene (lane Z-4). Samples (10 pmol) of unlabeled DNA from pUC19 (panel A, lane 4) or pAL364 (panel A, lane 5) were added to the binding mixture as competitor DNA. After addition of 20 μl of 50% glycerol solution to the reaction mixture, 15-μl portions of the reaction mixtures were charged in the slots of a 4% polyacrylamide gel and electrophoresed in low-ionic-strength buffer. The polyacrylamide gel was then dried and autoradiographed. The arrowheads in panel A indicate the retarded bands.

187-bp fragment (and pUC19 DNA) were labeled with ^{32}P, and the ^{32}P-labeled DNAs were incubated with 10 μg of the *E. coli* extract containing β-Gal::PHO4 protein in 40 μl of binding mixture. Though a significant amount of labeled DNAs remained at the origin due to nonspecific binding with an unidentified protein fraction in the *E. coli* extract harboring pUR290 (Z) (Fig. 4A, lane 2) or harboring the pUR291-*PHO4* plasmid (Z-4) (lanes 3, 4, and 5), three shift bands were detected after addition of the β-Gal::PHO4 fused protein (Fig. 4A, lane 3) but not on addition of β-galactosidase (lane 2). The densities of the shifted bands of the 187-bp DNA decreased significantly after addition of a 50-fold molar excess of unlabeled pAL364 DNA (lane 5) but not after addition of pUC19 DNA (lane 4) to the reaction mixture. These results indicate that the mobility shifts were due to sequence-specific binding of the β-Gal::PHO4 fused protein to the 132-bp *PHO8p* DNA.

In the same way, a ^{32}P-labeled 290-bp *Eco*RI-*Hin*dIII fragment bearing a 226-bp *PHO8p* DNA having the 44-bp distal regulatory region was prepared from pAL411 (Fig. 1B), and its binding with β-Gal::PHO4 was examined. No clear mobility shift was seen with this fragment (Fig. 4B).

We detected no similarities of these two sequences with the published UAS sequences of *PHO5* (4, 22, 27, 37) except the 5'-CACGT-3' motif suggested by Vogel et al. (37) at nucleotide positions −535 to −531 in the proximal 47-bp region but not in the distal 44-bp region (Fig. 3; details not shown).

For confirmation of the function of the 5'-CACGT-3' motif, three 15-, 20-, and 25-bp oligodeoxynucleotides with the same nucleotide sequences of 12-, 16-, and 20-bp, respectively, at and directly flanking the 5-bp motif of *PHO8p* with a 5' protruding end of *Bam*HI for the top strand and a *Sal*I protruding end for the bottom strand were synthesized (Fig. 5A). These three synthetic fragments were labeled with ^{32}P by filling in the two protruding ends, and the resulting double-stranded DNAs, having molecular sizes of 19, 24, and 29 bp, respectively, as a result of the fill-in reaction were used as probe DNAs in the gel retardation assay. All these DNAs gave clear triplet shift bands on reaction with β-Gal::PHO4 protein (Fig. 5B, lanes 3, 6, and 9). These findings indicate that the 12-bp sequence 5'-CCACGTGCAGCG-3', from positions −536 to −525 of *PHO8p*, is sufficient for binding PHO4 protein in vitro.

Expression of the *PHO8-HIS5* hybrid promoter. For examination of whether the DNA fragments bound with

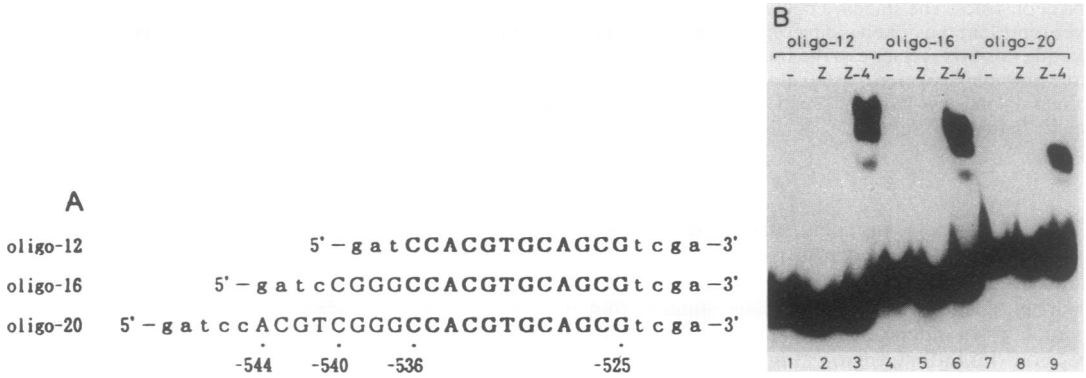


FIG. 5. Gel retardation assays with synthetic oligonucleotides bearing the 6-bp 5'-CACGTG-3' motif and its flanking region in *PHO8p* DNA. (A) Synthetic nucleotides used as probes with *Bam*HI and *Sal*I protruding ends on the top and bottom strand, respectively. These protruding ends of the probe DNAs were filled in with [α-^{32}P]dATP, dGTP, dCTP, and dTTP. The capital letters indicate the synthetic 12-bp (oligo-12), 16-bp (oligo-16), or 20-bp (oligo-20) nucleotides with the same sequences as authentic *PHO8p* DNA, and the lowercase letters represent linker sequences. (B) Separation of samples (0.3 pmol) of the labeled synthetic fragments treated with cell extracts of *E. coli* harboring pUR290 (Z; lanes 2, 5, and 8) or β-Gal::PHO4 (Z-4; lanes 3, 6, and 9) or without treatment (lanes 1, 4, and 7) on a polyacrylamide gel (15%).

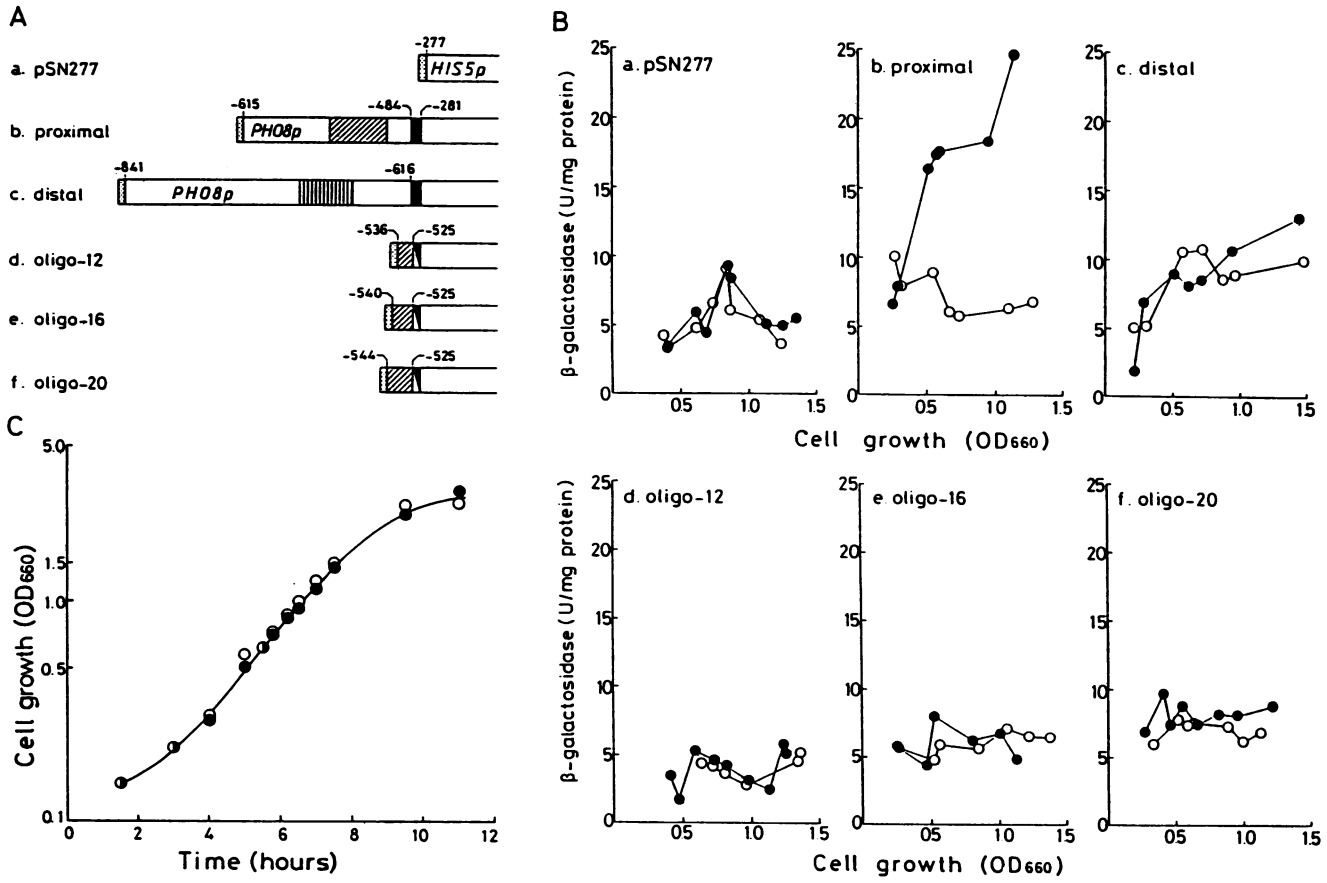


FIG. 6. Responses of chimeric promoters to P_i signals. (A) Construction and structure of *PHO8-HIS5* and synthetic oligonucleotide-*HIS5* chimeric promoters. A 3.6-kbp *Pst*I-*Xho*I fragment of pSN2 bearing the −281 (originally *Hin*fI, but ligated with an 8-bp *Xho*I linker; Fig. 1B) to +118 (originally *Ava*II but ligated with an 8-bp *Bam*HI linker) region was connected with the smaller *Pst*I-*Xho*I fragment of pAL447 (bearing the *PHO8p* DNA of the −615 to −484 region of *PHO8p* DNA; Fig. 1B) or pAL458 (bearing the −841 to −616 region of *PHO8p* DNA) or with similar *Pst*I-*Sal*I fragments of pAL459 (bearing oligo-12), pAL460 (oligo-16), and pAL461 (oligo-20). The control used was pSN277 bearing *HIS5p* DNA from −277 (originally the *Dde*I site, but ligated with an 8-bp *Bam*HI linker) to +118, constructed in a previous study (23). Smaller *Bam*HI fragments of these plasmid DNAs bearing composite promoters were prepared and ligated at the *Bam*HI site of the polycloning site of pSN616ΔB (Fig. 1B). The resultant composite plasmids were restricted at the *Kpn*I site in the *LEU2* DNA and used to transform *S. cerevisiae* K3-5D. Insertion of a single copy of the composite plasmid at the *leu2* locus in all the transformants was confirmed by Southern blotting after digestion of genomic DNAs with *Bgl*II and hybridized with ^{32}P-labeled YIp33 DNA as a probe. The diagonally hatched boxes indicate the proximal regulatory region of *PHO8p* or synthetic oligonucleotides, and the vertically hatched box indicates the distal regulatory region. The stippled boxes at the end of each promoter DNA indicate the *Bam*HI linker, and the solid boxes indicate *Xho*I linkers. The half-solid boxes indicate *Sal*I-*Xho*I junctions. Numbers above the *PHO8* and *HIS5* DNAs represent nucleotide positions relative to the translation initiation codon. (B) Time course of change in β-galactosidase activity in transformants. Transformants of *S. cerevisiae* K3-5D with inserts of single copies of the above composite plasmids at the *leu2* locus were cultivated in YPD medium at 30°C for 2 days. The cells were then harvested, washed, and inoculated into YPD medium of high P_i (○) or low P_i (●) to give an initial OD_{660} of 0.1. The cultures were shaken gently at 30°C and assayed for β-galactosidase activity at intervals. (C) Cell growth of strain K3-5D with inserts of composite DNA in the proximal region. Other transformants showed essentially the same cell growths. Symbols: Same as in panel B.

β-Gal::PHO4 protein in vitro are able to sense the P_i signals, the various *PHO8p-HIS5'-'lacZ* chimeric DNAs shown in Fig. 6A were constructed. The resulting plasmids were then integrated into the *leu2* locus of *S. cerevisiae* K3-5D. K3-5D cells in which a single copy of the DNA construct was integrated were cultivated in 200 ml of the low-P_i or high-P_i version of YPD medium at 30°C, and their β-galactosidase activities were determined at appropriate intervals. Typical examples of replicate experiments (at least three) are shown in Fig. 6B. The results were highly reproducible. The transformant with the intact *HIS5'-'lacZ* construct did not respond to P_i signals (Fig. 6B, panel a). In contrast, the hybrid promoter connected with the 132-bp *PHO8p* DNA, bearing the proximal regulatory region, expressed significantly higher β-galactosidase activity in low-P_i medium than in high-P_i medium (panel b). On the other hand, the *HIS5* promoter DNA connected with the 226-bp *PHO8p* DNA from nucleotide positions −841 to −616, bearing the distal regulatory region, did not sense P_i signals (panel c), although this region enhanced the rALPase activities, as shown in Fig. 2B. These results suggest that the distal regulatory region is not directly concerned with sensing P_i signals. None of the composite promoters with synthetic oligonucleotides derepressed β-galactosidase expression in low-P_i medium (panels d, e, and f). These findings indicate that the 132-bp region from −615 to −484 bearing the 47-bp proximal

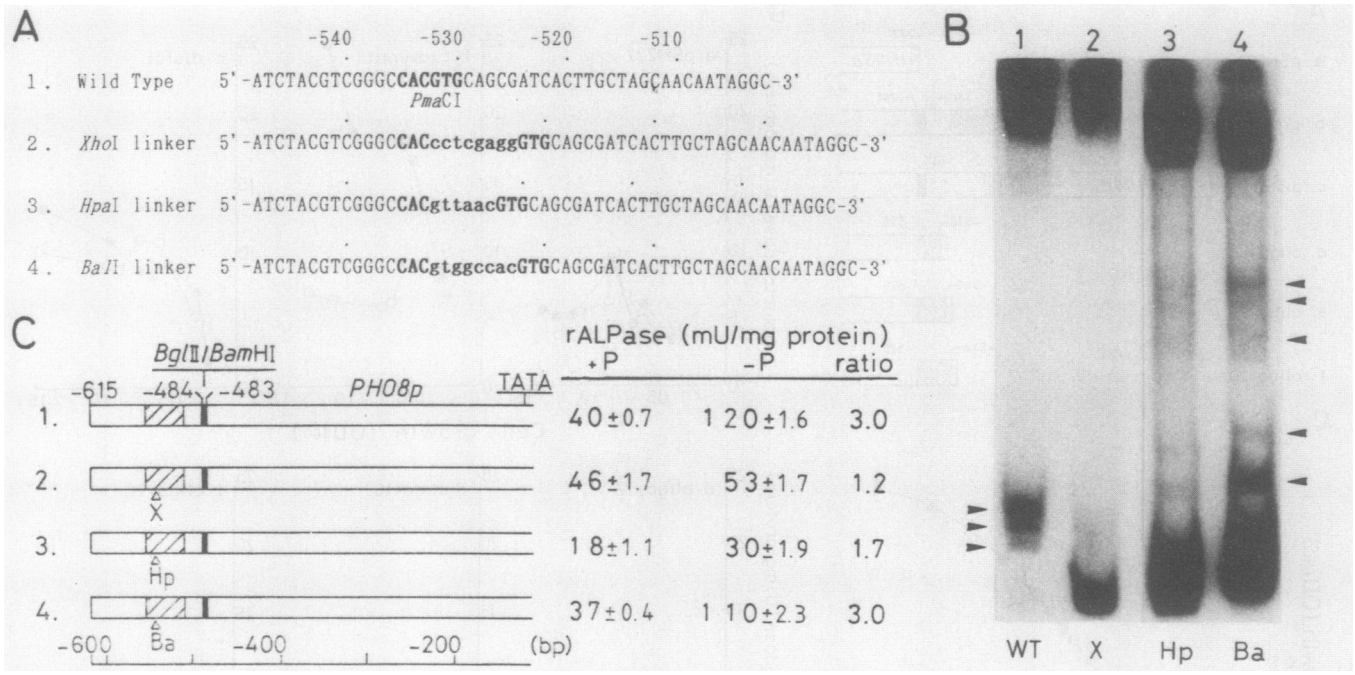


FIG. 7. Effect of linker insertions on the *Pma*CI site in the proximal region. (A) Sequences of the 47-bp proximal regulatory region bearing various linker insertions. An *Xho*I, *Hpa*I, or *Bal*I linker (lowercase letters) was inserted at the *Pma*CI site of the 47-bp region of *PHO8p* in the 187-bp *Eco*RI-*Hin*dIII fragment of pAL364 (Fig. 1B). (B) Gel retardation assays of the 187-bp fragments with or without insertion of the *Xho*I, *Hpa*I, and *Bal*I linkers at the *Pma*CI site. Each binding mixture (40 μl) contained 10 μg of protein of the β-Gal::PHO4 preparation and 0.3 pmol of probe DNA labeled with ^{32}P as described in the legend to Fig. 4. The probe DNAs in the reaction mixtures were the intact 187-bp *Eco*RI-*Hin*dIII fragment of pAL364 (lane WT) and those with the *Xho*I linker (lane X), *Hpa*I linker (lane Hp), and *Bal*I linker (lane Ba) insertions. Arrowheads indicate retarded bands. (C) rALPase activities with the *PHO8* gene bearing wild-type or modified promoters with various linker insertions. Enzyme activities were determined as described in the legend to Fig. 2. Each value represents the average of at least three independent experiments on a number of different clones having the same DNA insertions at the *PHO8* locus. The hatched boxes indicate the proximal regulatory region without or with insertion of the *Xho*I (X), *Hpa*I (Hp), or *Bal*I (Ba) linker (Δ). Numbers above the bar representing *PHO8p* DNA indicate nucleotide positions relative to the translation initiation codon.

regulatory sequence is sufficient for sensing P_i signals, whereas the 20-bp region from positions −545 to −526 is not.

Inactivation of the regulatory region by linker insertion. Since the 5′-CACGT-3′ sequence at −535 overlaps the restriction sequence for *Pma*CI, 5′-CACGTG-3′, we inserted various linkers at this blunt-ended restriction site (nucleotides −533 and −532 [Fig. 7A]) of the 132-bp *Hin*cII fragment of the *PHO8p* DNA prepared from pAL364 (Fig. 1B). The 187-bp *Eco*RI-*Hin*dIII fragments bearing the 132-bp fragment with various inserted linkers were tested for ability to bind the β-Gal::PHO4 protein by the gel retardation assay. No mobility shift was observed with the DNA with an insertion of an 8-bp *Xho*I linker (Fig. 7B, lane 2). Insertions of the 6-bp *Hpa*I linker (5′-GTTAAC-3′) and 8-bp *Bal*I linker (5′-GTGGCCAC-3′) at the *Pma*CI site created an inverted and direct repeat, respectively, of the 5′-CACGT-3′ sequence (Fig. 7A). These two insertions resulted in several slowly moving bands (Fig. 7B, lanes 3 and 4, indicated by arrowheads). This result suggests that the PHO4 binding sites were increased by the *Hpa*I and *Bal*I linker insertions. Since the binding signals of these modified DNAs were lower than that of the wild-type DNA (lane 1), inverted or tandem arrangement of the 5-bp motif side by side resulted in lower affinity for PHO4 protein or an unstable binding complex owing to too short a distance between the two binding motifs.

For examination of the effect of linker insertions at the *Pma*CI site on the promoter function of the proximal regulatory region of *PHO8p*, pAL364 (Fig. 1B) and the modified plasmids were restricted at the *Sal*I site at position −486 in *PHO8p* DNA, ligated with the *Bgl*II linker, and recircularized. The plasmid DNAs were propagated in *E. coli*, and then the 150-bp *Bam*HI-*Bgl*II fragment bearing the wild-type 132-bp *PHO8p* DNA and the corresponding fragments of the modified pAL364 were prepared. These fragments were ligated to the YIp33-based chimeric plasmid at the *Bam*HI end of the *PHO8p* DNA, in which the upstream region from position −483 was deleted (Fig. 2A). The chimeric plasmids with the DNA constructs, whose promoter regions are shown in Fig. 7C, were inserted at the *PHO8* locus of *S. cerevisiae* K143-1C similarly to the constructs shown in Fig. 2. The transformants were cultivated in high-P_i and low-P_i versions of Leu test medium, and their rALPase activities

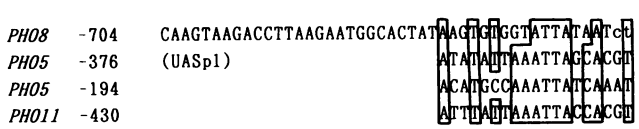


FIG. 8. Comparison of the distal regulatory region of *PHO8p* with some 5′ upstream regulatory regions of *PHO5* and *PHO11* reported by Hinnen et al. (9, 27). Numbers indicate nucleotide positions from the relevant ATG codon. Lowercase letters in the *PHO8* sequence indicate nucleotide sequences outside the 44-bp distal regulatory region. Homologous nucleotides are boxed.

were determined. The composite *PHO8p* DNA bearing the wild-type 132-bp sequence and that with the inserted *Bal*I linker showed normal regulatory function (Fig. 7C). The fragment bearing the *Hpa*I linker conferred lower rALPase activity than the wild-type fragment but sensed P_i signals. The fragment with the *Xho*I linker insert, however, did not derepress rALPase activity. These observations are in accord with the results of gel retardation experiments (Fig. 7B) and suggest that the 5′-CACGTG-3′ sequence is more efficient than the 5′-CACGTT-3′ sequence.

DISCUSSION

By deletion analysis of *PHO8* DNA (Fig. 2), the sites necessary to derepress *PHO8* were found to be located in two regions: a 47-bp region between positions −548 and −502 and a 44-bp region between positions −704 and −661 (Fig. 3). Another region between −421 and −289 may contain an inhibitory sequence for *PHO8* expression. The 5-bp 5′-CACGT-3′ motif or the 6-bp 5′-CACGTG-3′ motif located in the proximal region was found to be essential for binding the β-Gal::PHO4 protein (Fig. 5 and 7). The 6-bp 5′-CACGTG-3′ motif seems to be more favorable than 5′-CACGTT-3′ for *PHO8* gene expression (Fig. 7C). This 6-bp sequence is similar to the consensus sequence 5′-NNCANNTG-3′, where N is any nucleotide, proposed as a binding sequence for a protein bearing the helix-loop-helix motif of the DNA-binding domain (5), which PHO4 protein has (24). In the 5′ upstream region of the *PHO81* and *PHO84* genes, transcription of which is also coregulated with *PHO8* and *PHO5* (40, 42), we found one and three copies, respectively, of the 6-bp motif (unpublished data).

The 6-bp motif is, however, not sufficient alone for *PHO8* regulation, because a 20-bp synthetic oligonucleotide containing the 6-bp motif could not sense P_i signals in vivo (Fig. 6). The 132-bp *PHO8p* DNA from positions −615 to −484 bearing the 47-bp proximal region clearly bound with β-Gal::PHO4 protein (Fig. 4) and could sense P_i signals (Fig. 6). These findings, in addition to the results of deletion experiments (Fig. 2), strongly suggest that the 47-bp region fulfills the basic requirements for *PHO8* regulation.

The distal region conferred regulatory function when it was connected with a *PHO8* DNA, even when its proximal region was deleted but the promoter sequence from position −365 to the ATG codon was retained (Fig. 2B). The distal region, which does not contain any sequence homologous to the 6-bp motif, has significant similarities with the 19-bp dyads suggested as the UAS elements in *PHO5p* DNA (9, 27). These 19-bp dyads in *PHO5p* have similarities to each other when the actual nucleotide sequences were converted into the purine-pyrimidine notation (9). One of the three proposed 19-bp dyad UAS*p* sequences at nucleotide positions −194 to −176 could not bind PHO4 protein (37), but deletion of this region slightly decreased *PHO5* expression (9, 27). A portion of the nucleotide sequence in the distal regulatory region of *PHO8p* has some similarities with this 19-bp sequence of *PHO5p* (Fig. 8). The homologous regions in these two sequences also have similarities with the 19-bp dyad of UASp1 in *PHO5p*, located at positions −376 to −358, which can bind the PHO4 protein (37) and is suggested to play a central role in *PHO5* regulation (1, 37), but no similarities were detected with another 19-bp dyad, UASp2, of *PHO5p* located at −254. We also found some similarities of the putative 19-bp UASp sequence of *PHO11* (9, 27), another rAPase gene (17, 32), with the above homologous sequences (Fig. 8). These sequences may stabilize the tertiary structure of DNA or the chromatin structure in the promoter region in low-P_i medium or may be involved in its function in the next step of the PHO4 binding. Although the 133-bp inhibitory region also contains a sequence similar to the 19-bp dyads in the purine-pyrimidine notation, no such similarities with the sequences shown in Fig. 8 were detected.

In assays of binding of the β-Gal::PHO4 protein with the 132-bp fragment of the *PHO8p* DNA and the synthetic oligonucleotides, we consistently observed triplet bands (Fig. 4 and 5). Vogel et al. (37) reported similar findings in assays of binding of *PHO5p* DNA with PHO4 protein. These findings were explained by oligomerization of PHO4 protein in a previous study (24).

ACKNOWLEDGMENTS

We thank E. Hiraoka and Z. Kuromitsu of this laboratory for their technical assistance.

This study was supported by Grants-in-Aid for Special Project Research (grant 61128005) and for General Scientific Research (grant 60480058) to Y.O. from the Ministry of Education, Science, and Culture of Japan.

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