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Transcriptional and Post-Transcriptional Control of *PHO8* Expression by *PHO* Regulatory Genes in *Saccharomyces cerevisiae*

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A DNA fragment bearing the *PHO8* gene, which encodes repressible alkaline phosphatase of *Saccharomyces cerevisiae*, was cloned. Northern hybridizations with the *PHO8* DNA as probe indicated that the *PHO8* transcript is 1.8 kilobases in length and is more abundant in cells grown in low-phosphate medium than in high-phosphate medium. The *pho9* mutant, whose phenotype is defective in the activity of repressible alkaline phosphatase, produced as much of the *PHO8* transcript as did the *PHO9*$^+$ cells. Hence, the *PHO9* product should act at the post-transcriptional level. The *pho4* mutant could not derepress the *PHO8* transcript, whereas the *pho80* mutant could, irrespective of the amount of P_i in the medium, as has been suggested by genetic study.

Cellular extracts of *Saccharomyces cerevisiae* contain two species of enzymes that hydrolyze *p*-nitrophenylphosphate at alkaline pH (2, 10, 26): one is specific *p*-nitrophenylphosphatase (*p*-NPPase); the other is a nonspecific, repressible alkaline phosphatase (rALPase; EC 3.1.3.1). Specific *p*-NPPase is produced constitutively (26) and has a molecular weight of ca. 60,000 (1). rALPase is a glycoprotein with a molecular weight of ca. 130,000 and is composed of two subunits, each with a molecular weight of 66,000 (18). This enzyme is encoded by the *PHO8* gene on chromosome IV (15) and is localized in the cell vacuole (7). Expression of *PHO8* is thought to be regulated by a system consisting of products of the *PHO4*, *PHO80*, *PHO81*, *PHO85*, and *PHO9* genes under the influence of P_i in the medium (19). The *PHO4* and *PHO81* gene products are indispensable for the full derepression of *PHO8* at the transcriptional stage, and the products of the *PHO80* and *PHO85* genes inhibit the function of the *PHO4* product. The *pho9* mutant is defective in the activity of rALPase and has a sporulation-defective phenotype in a *pho9*/*pho9* diploid (15). It was shown, however, that *PHO9* is identical to the *PEP4* gene (15). Since the *PEP4* product is supposed to be required for the conversion of the inactive precursor to the active mature enzyme of at least four vacuolar hydrolases, proteinases A and B, carboxypeptidase Y, and RNase, along with rALPase (14, 29), the *PHO9* product may not be involved in the transcriptional control of *PHO8*. This communication deals with the examination of these alternatives by detection of the *PHO8* transcript by Northern hybridization, with the cloned *PHO8* DNA as probe.

A yeast gene bank with *Escherichia coli* JA221 (F$^-$ *leuB6* *ΔtrpE5 lacY recA1 hsdM*$^+$ *hsdR*) (8) as the host was constructed by connecting the *Sau*3A fragment of chromosomal DNA of strain P-28-24C (*MAT***a** *pho3-1* and prototrophic) to the *Bam*HI site of YEp13 (10.7 kilobases [kb]; a chimeric plasmid consisting of pBR322, the *LEU2* gene of *S. cerevisiae*, and the replication origin of 2μm DNA [4]). The conditions for cell cultivation in L broth and for transformation of *E. coli* (27) and general methods for the recombinant DNA experiments (25) were as described previously. Plasmid DNAs were prepared by the method of Clewell and Helinski (9) from a portion of the gene bank and used to transform *S. cerevisiae* NA79-10C (*MATα pho8-2 leu2 his3 trp1 can1*) to the leucine prototrophic phenotype (Leu$^+$) with the protoplasted yeast cells as described previously (27). All of the *S. cerevisiae* strains used were constructed by crossing the transformation-competent strains AH22 (12) and D13-1A (23) with the *pho8* and *pho9* mutants (15) or other *pho* mutants originated from P-28-24C and F16C (*MATα pho3-1* prototrophic, a mating-type convertant of P-28-24C) (24, 28). Leu$^+$ yeast transformants appeared on the Leu test medium (27) and were replicated onto minimal low-P_i medium (15) supplemented with tryptophan and histidine, and then were screened for the activity of alkaline phosphatase by the staining method (15). For detection of the activity of rALPase only (but not specific *p*-NPPase), soft agar containing 0.5 mg of α-napththylphosphate, instead of *p*-nitrophenylphosphate, and 5 mg of Fast Red TR salt (color index no. 37085; Sigma Chemical Co., St. Louis, Mo.) per ml was used. We observed various alkaline phosphatase-productive (Alp$^+$; repressible ALPase, rAlp$^+$, in this case) colonies. Total cellular DNAs prepared from two of these rAlp$^+$ clones by the method of Cameron et al. (5) were used for purification of the plasmid clones by successive transformation of *E. coli* and the yeast host, NA79-10C (*pho8 leu2*; only the relevant genotype is described hereafter). We picked up 15 yeast Leu$^+$ clones in the final transformants and found that all of them showed the rAlp$^+$ phenotype. A plasmid, designated pAL101, was isolated from one of these clones.

The restriction maps of pAL101 and the derivative plasmids are shown in Fig. 1. The *PHO8* gene in pAL101 was delimited to the 2.8-kb *Bam*HI/*Sau*3A-*Eco*RI region by partial digestion with *Eco*RI or *Hin*dIII and religation, or subcloning of the *Eco*RI or *Sal*I fragment of pAL101 in YEp6 (7.9 kb, bearing the *HIS3* gene of *S. cerevisiae* [3]), and transformation of NA79-10C (*pho8-2 leu2 his3*). Since pAL125 could not confer the rAlp$^+$ phenotype, the *PHO8* gene must be located at the left end of the 2.8-kb fragment.

The *pho8* mutant, NA79-10C, was complemented by pAL101 or pAL109 (a derivative plasmid of pAL101; Fig. 1) and showed significant levels of activities on both *p*-nitrophenylphosphate and α-naphthylphosphate (Table 1). The

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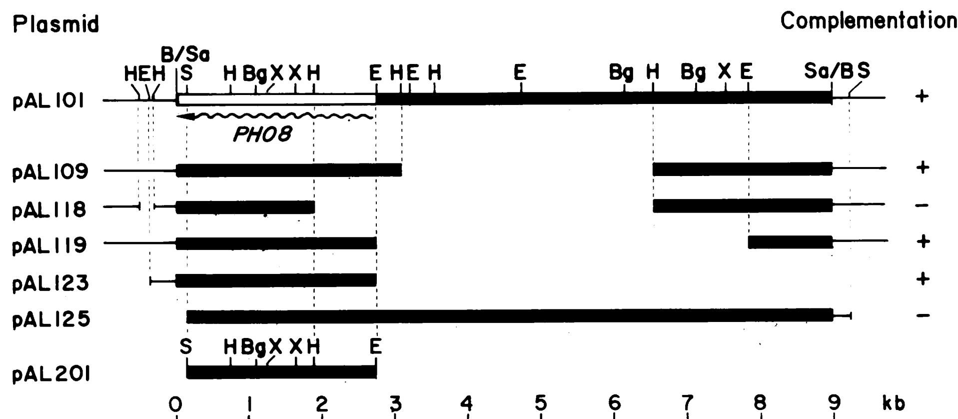

FIG. 1. Restriction maps of the *S. cerevisiae* DNA fragments cloned on YEp13 and their complementation of the *pho8-2* mutation. Thin lines indicate the YEp13 DNA, and thick lines (with open box) are the cloned *S. cerevisiae* DNA fragments. Open box indicates the delimited region coding for the *PHO8* gene. The wavy line with the arrowhead at the *PHO8* region on pAL101 indicates the transcriptional direction of *PHO8*. Plasmids pAL109, pAL118, and pAL119 were derived from pAL101 by deleting a small fragment by digestion with *Hin*dIII or *Eco*RI and religation. pAL123 and pAL125 were obtained by subcloning the indicated *Eco*RI or *Sal*I fragment of pAL101 in YEp6 as the vector. Plasmid pAL201 was constructed by subcloning the indicated fragment in pBR322. Restriction sites shown are B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sa, *Sau*3A; and X, *Xho*I. B/Sa or Sa/B indicates the joint site of *Bam*HI and *Sau*3A cohesive ends.

transformants with pAL109, however, showed threefold-higher activities than those with pAL101 for both the substrates, and the activity levels were much higher than those of the wild-type strain. We do not know the reason for this phenomenon. Unexpectedly, the rAlp$^-$ phenotype of the *pho9* mutant, K7-6B (*MAT*α *pho9-1 leu2-3,112 trpl canl*), was also complemented with pAL101 and pAL109 (Table 1). Another *pho9* phenotype, Spo$^-$, was not, however, restored by the pAL101 or pAL109 transformation, and all the transformants examined were found to lack colonial carboxypeptidase Y activity (data not shown). Although significant increases in the enzyme activities toward both the substrates were shown in the pAL109 transformants of the *pho9* mutant (the reasons for this will be discussed later), these observations strongly suggest that pAL101 and pAL109 contain the *PHO8* gene.

That pAL101 contains *PHO8* was confirmed by the integration of the pAL101 DNA into the yeast chromosome. We obtained two yeast clones in which pAL101 was integrated spontaneously into a chromosome during the curing experiments of NA79-10C [*MAT*α *pho8-2 leu2*(pAL101)] by cultivating it in nutrient medium. They were crossed with M49-5D (*MAT***a** *pho8-2 leu2-3,112 ade8 rna3*), and the resultant diploids were subjected to tetrad analysis. In both crosses, the Leu phenotype showed a 2+:2− segregation, as did the Rna (tested by temperature-sensitive growth on a nutrient plate at 35°C) and Ade (adenine auxotrophic) phenotypes. The pooled tetrad data of the two resultant diploids showed a tetrad distribution of 36:0:3 in the ratios of parental ditype, nonparental ditype, and tetratype asci for the Leu and Rna phenotypes and 30:0:9 for the Leu and Ade phenotypes. Thus, pAL101 might be integrated into chromosome IV at a site 4 centimorgans (cM) from the *rna3* locus and 12 cM from the *ade8* locus by the equation of Perkins (20). Since our previous data showed 8 cM between *pho8* and *rna3*, and 30 cM between *pho8* and *ade8* in the gene order of *ade8-rna3-pho8* on chromosome IV (15), the results described above indicate that pAL101 is integrated at or close to the *pho8* locus. Thus, we concluded that pAL101 bears the *PHO8* gene.

TABLE 1. Drepressed level of alkaline phosphatase activity of the transformants

Strain	Genotype[a]	Enzyme activity (U/mg of protein)[b] on:	
		p-Nitrophenyl-phosphate	α-Naphthyl-phosphate
P-28-24C	Wild type	0.32	0.18
NA79-10C	*pho8-2*	0.07	0.00
K7-6B	*pho9-1*	0.08	0.00
NA79-10C(pAL101)	*pho8-2*(pAL101)	0.16	0.07
NA79-10C(pAL109)	*pho8-2*(pAL109)	0.53	0.24
K7-6B(pAL101)	*pho9-1*(pAL101)	0.08	0.04
K7-6B(pAL109)	*pho9-1*(pAL109)	0.43	0.19

[a] Only relevant genotypes are indicated.
[b] Crude cell extracts were prepared from the cells grown on minimal low-P_i medium supplemented with appropriate nutrients at 30°C for 15 to 18 h with shaking. The enzyme assay was performed at 30°C as described previously (15), with *p*-nitrophenylphosphate or α-naphthylphosphate as substrate. The protein concentration of the cell extract was determined by the method of Lowry et al. (16). One unit of alkaline phosphatase was defined as the amount of enzyme that liberated 1 μmole of *p*-nitrophenol or P_i per min.

To test whether the expression of the *PHO8* gene in plasmid pAL109 is affected by the *PHO* regulatory genes in the same way as the *PHO8* gene on the chromosome (19), we examined the rALPase productivity of the *pho4 pho8*, *pho80 pho8*, and *pho85 pho8* double mutants, and the *pho8* mutant harboring pAL109. The transformants generally showed slightly higher activities than the wild-type strain, perhaps due to the dosage effect of the *PHO8* gene on the plasmid. The *pho4 pho8* double mutant carrying pAL109 showed no increment of rALPase activity under derepressed conditions, whereas the *pho80 pho8* and *pho85 pho8* double mutants carrying pAL109 produced a high activity level even under repressed conditions (Table 2). Thus, the expression of *PHO8* on the plasmid is under the control of the *PHO* regulatory genes, and the cloned fragment is able to respond to the regulatory signals.

To test the alternative mechanisms of whether the *PHO9* (= *PEP4*) gene product is involved in the transcriptional control of *PHO8* as described previously (19), or in the conversion of the rALPase precursor to the mature active enzyme (11, 14, 29), we investigated the *PHO8* transcript semi-quantitatively by Northern hybridization, using the

TABLE 2. Alkaline phosphatase activity of the cells having *pho4*, *pho80*, or *pho85* mutation carrying plasmid pAL109[a]

Strain[b]	Genotype	Enzyme activity (U/ml per OD_{660})[c] in:	
		High-P_i medium	Low-P_i medium
P-28-24C	Wild type	0.03	0.12
K38-8A	*pho8-2 pho4-1*(pAL109)	0.08	0.08
K38-8B	*pho8-2 PHO4*$^+$(pAL109)	0.08	0.14
K39-3A	*pho8-2 pho80-22*(pAL109)	0.12	0.23
K39-3B	*pho8-2 PHO80*$^+$(pAL109)	0.06	0.15
K40-1D	*pho8-2 pho85-22*(pAL109)	0.20	0.20
K40-1A	*pho8-2 PHO85*$^+$(pAL109)	0.08	0.17

[a] Cells of each strain were precultured in 5 ml of minimal low-P_i medium supplemented with 20 mg each of L-histidine and L-tryptophan per ml for 24 h. A 0.2-ml portion of the preculture was inoculated into 5 ml of minimal high-P_i or low-P_i medium supplemented with histidine and tryptophan, and the cells were cultivated for 15 h at 30°C. rALPase activity was assayed at 30°C with permeabilized cells as the enzyme source (26) and *p*-nitrophenylphosphate as substrate.

[b] Strains K38-8A and K38-8B are the meiotic segregants from the cross of NA79-10C (*MATα PHO4*$^+$ *pho8-2 leu2*)(pAL109) × K32-2C (*MATa pho4-1 pho8-2 leu2*); strains K39-3A and K39-3B are the meiotic segregants from the cross of NA79-10C (*MATα PHO80*$^+$ *pho8-2 leu2*)(pAL109) × K33-7B (*MATα pho80-22 pho8-2 leu2*); and strains K40-1D and K40-1A are meiotic segregants from the cross of NA79-10C (*MATα PHO85*$^+$ *pho8-2 leu2*)(pAL109) × K34-7C (*MATa pho85-22 pho8-2 leu2*).

[c] OD_{660}, Units of optical density at 660 nm.

^{32}P-labeled DNA of pAL201 or of the 1.1-kb *Sal*I-*Xho*I fragment of pAL201 (Fig. 1) as probe. Total cellular RNAs were prepared as described by Jensen et al. (13) from the cells of the wild-type strain, P-28-24C; the *pho9* mutant, AL212-4D (*MATa pho3-1 pho9-1 ade10*); the *pho4* mutant, P-144-2D (*MATα pho3-1 pho4-1 arg6*); and the *pho80* mutant, P-146-8B (*MATα pho3-1 pho80-1 arg6*), each grown on nutrient high-P_i and low-P_i media to the logarithmic phase. A 1.8-kb transcript appeared in both of the RNA samples prepared from cells grown in high-P_i and low-P_i, with the same patterns as for the wild-type strain (Fig. 2) and the *pho9* mutant (Fig. 2A). These results indicate that the *PHO9* gene is not concerned with the transcription of *PHO8*. The intensities of the hybridized bands of low-P_i samples were, however, much stronger than those of high-P_i samples. The *pho4* mutant, in which rALPase is not derepressed, produced only a basal amount of the transcript even in cells grown in low-P_i medium, whereas the amount of the *PHO8* transcript produced by the *pho80* mutant grown in high-P_i medium was similar to that from the same mutant grown in low-P_i medium (Fig. 2B). Thus, the *PHO4* and *PHO80* genes are involved in the regulation of *PHO8* transcription. The remaining two regulatory genes, *PHO81* and *PHO85*, were not tested, but they should be included in the same regulatory system as *PHO4* and *PHO80*, because genetic data show that *PHO81* and *PHO85* are hypostatic to the *PHO4* gene (see review in reference 19). However, the hybridization data for the *pho9* mutant support the idea that the *PHO9* product is required for the post-transcriptional processing of the *PHO8* products or the rALPase precursor. Thus, our original model (19) should be revised.

The transcriptional direction of the *PHO8* gene was determined by Northern hybridization with the ^{32}P-labeled *PHO8* DNA prepared by strand-specific labeling. pAL201 (Fig. 1) was linearized by cutting it at the *Bgl*II site in the 2.6-kb *Eco*RI-*Sal*I yeast fragment (pAL201 has only one site for *Bgl*II). Then strand-specific labeling with [^{32}P]dATP, using T4 DNA polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan), was performed by the method of O'Farrell (cited in Mainatis et al. [17]) for both strands at the 3′ end of the linear pAL201 molecule. The labeled DNA was digested with *Sal*I and *Xho*I simultaneously, and the 0.95-kb *Sal*I-*Bgl*II and 0.4-kb *Xho*I-*Xho*I fragments were fractionated by polyacrylamide gel electrophoresis. If the *PHO8* gene is transcribed from the *Eco*RI to the *Sal*I site, the 0.95-kb *Sal*I-*Bgl*II fragment should hybridize with the 1.8-kb *PHO8* transcript; but if it is transcribed in the opposite direction, the trancript should hybridize with the 0.4-kb *Xho*I-*Xho*I fragment. Polyadenylated [poly(A)$^+$] RNA prepared from the wild-type strain P-28-24C was hybridized with the two probes described above. The results showed that the *PHO8* transcript hybridized with the 0.95-kb *Sal*I-*Bgl*II fragment but not with the 0.4-kb *Xho*I-*Xho*I fragment (Fig. 3). Thus, the *PHO8* gene is transcribed leftward (Fig. 1).

The data of Northern hybridization (Fig. 2) clearly indicated that the *PHO9* function is at the post-transcriptional

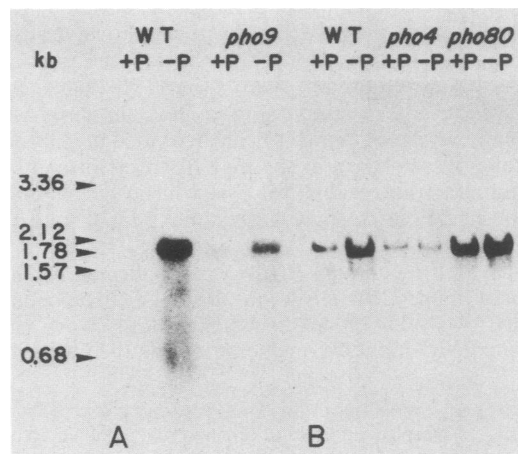

FIG. 2. Northern hybridization of poly(A)$^+$ RNA prepared from the wild-type or *pho9*, *pho4*, and *pho80* mutant cells grown in nutrient high P_i or nutrient low-P_i medium with ^{32}P-labeled DNAs bearing the *PHO8* gene as probe. Poly(A)$^+$ RNA was purified from the total cellular RNA by oligodeoxythymidylate-cellulose (type 7; P-L Biochemicals, Inc., Milwaukee, Wis.) affinity chromatography (22). Poly(A)$^+$ RNA was dissolved in 50% (vol/vol) formamide–6% (vol/vol) formaldehyde in 10 mM sodium phosphate buffer (pH 6.8), and the solution was heated at 65°C for 5 min and quickly cooled. Samples (5 μg for each slot) were electrophoresed on a 1% agarose gel containing 5% formaldehyde–1 mM EDTA in 10 mM sodium phosphate buffer (pH 6.8), transferred onto nitrocellulose filters, and hybridized with either ^{32}P-labeled pAL201 DNA prepared by nick translation (21) (A), or the 1.1-kb *Sal*I-*Xho*I fragment of pAL201 (B), as probe. Symbols: WT, the wild-type strain P-28-24C; *pho9*, strain AL212-4D; *pho4*, strain P-144-2D; *pho80*, strain P-146-8B; +P, poly(A)$^+$ RNA prepared from the cells grown in nutrient high-P_i medium; −P, poly(A)$^+$ RNA from cells grown in nutrient low-P_i medium. Molecular sizes indicated on the left were obtained from the *Rsa*I fragments of pBR322 (17).

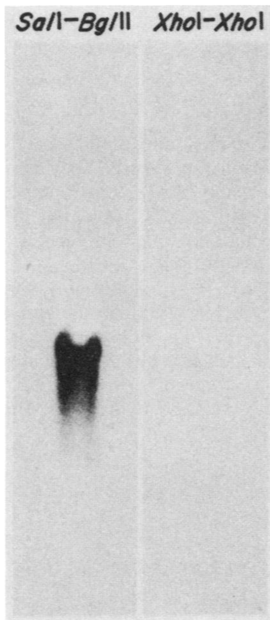

FIG. 3. Northern hybridization of the *PHO8* transcript with DNAs prepared by strand-specific labeling as the probe. Poly(A)$^+$ RNA prepared from the cells of the wild-type strain, P-28-24C, grown on nutrient low-P_i medium was treated as described in the legend to Fig. 2 and hybridized with ^{32}P-strand-specific labeled DNAs, the *Sal*I-*Bgl*II (ca. 5×10^7 cpm/μg of DNA) and *Xho*I-*Xho*I (ca. 4×10^7 cpm/μg of DNA) fragments, prepared from pAL201 (Fig. 1) as described in the text.

level of the *PHO8* expression. The question then arises of how to reconcile these facts with the finding, shown in Table 1, that the *pho9* mutation is complemented by the cloned *PHO8* gene. The most plausible explanation is that the *PHO8* gene on the plasmid is changed during the cloning so that the *PHO9* function is no longer needed for the primary translation product of the cloned *PHO8* gene to have an active configuration. In this context, the characteristics of the cloned *PHO8* gene are suggestive. From the location (Fig. 1) and transcriptional direction (Fig. 3) of the *PHO8* gene on the cloned fragment, it is possible to speculate that the 3′-terminal region of the *PHO8* gene is missing and the C-terminal region of the rALPase precursor might be truncated or modified. However, Hemmings et al. (11) suggested that the maturation event of carboxypeptidase Y precursor might be scission of the N terminal from the precursor protein, although they could not exclude the possibility of C-terminal scission. To reach a conclusion, it is necessary to clone and characterize the *PHO8* gene with a sufficiently long 3′-noncoding region.

A distinctive feature of *PHO8* gene regulation is the high level of basal activity of cells in the repressed condition in comparison with that of acid phosphatase encoded by the *PHO5* gene (26). An analogous phenomenon was found in the expression of the *SUC2* gene (6): the *SUC2* gene produces two species of transcripts, 1.8- and 1.9-kb RNAs, in derepressed conditions, but only the 1.8-kb RNA in the cells under repression by glucose. It was proposed that the transcription of the *SUC2* gene is initiated at two different sites depending on the cultural conditons: the 1.8-kb RNA, which encodes the intracellular, nonglycosylated invertase, is produced constitutively, and transcription of the 1.9-kb RNA, which encodes the secreted, glycosylated enzyme, is sensitive to glucose repression. In contrast, the *PHO8* gene apparently produced single species of mRNA under repressed and derepressed conditions (Fig. 2), though it is necessary to analyze the starting site of the transcripts.

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