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Regulation of Repressible Acid Phosphatase Gene Transcription in *Saccharomyces cerevisiae*

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We examined the genetic system responsible for transcriptional regulation of repressible acid phosphatase (APase; orthophosphoric-monoester phosphohydrolase [acid optimum, EC 3.1.3.2]) in *Saccharomyces cerevisiae* at the molecular level by analysis of previously isolated and genetically well-defined regulatory gene mutants known to affect APase expression. These mutants identify numerous positive- (*PHO4*, *PHO2*, *PHO81*) and negative-acting (*PHO80*, *PHO85*) regulatory loci dispersed throughout the yeast genome. We showed that the interplay of these positive and negative regulatory genes occurs before or during APase gene transcription and that their functions are all indispensible for normal regulation of mRNA synthesis. Biochemical evidence suggests that the regulatory gene products they encode are expressed constitutively. More detailed investigation of APase synthesis is a conditional *PHO80*(Ts) mutant indicated that neither *PHO4* nor any other protein factor necessary for APase mRNA synthesis is transcriptionally regulated by *PHO80*. Moreover, in the absence of *PHO80*, the corepressor, presumed to be a metabolite of P_i, did not inhibit their function in the transcriptional activation of APase.

Five principal enzymes perform the basic cellular housekeeping functions of phosphorus acquisition and metabolic integration of P_i in *Saccharomyces cerevisiae*. These are exocellular acid phosphatase (APase; orthophosphoric-monoester phosphohydrolase [acid optimum, 3.1.3.2.]), a phosphodiesterase with broad substrate specificity (20); a phosphate permease (18); polyphosphate kinase that converts intracellular P_i via ATP into vacuolar deposits of polyphosphate (7, 21, 29); and two enzymes, alkaline phosphatase (AlkPase) and polyphosphatase, located in the yeast vacuole, which hydrolyze polyphosphate (7, 9). These enzymes regulate intracellular concentrations of P_i and maintain cellular homeostasis (7, 8) by a cyclic pathway of polyphosphate synthesis and degradation (7). They are themselves regulated by external growth concentrations of P_i (6, 7, 9, 10).

This family of enzymes is a dispersed system composed of numerous structural and regulatory genes (Table 1). There are four known structural genes encoding APase (*PHO3*, *PHO5*, *PHO10*, *PHO11*), genes for AlkPase (*PHO8*) and P_i transport (*PHO84*), and eight genes affecting their expression (9, 23–28). Recessive mutations in *PHO4* and *PHO81* block the derepression of repressible APase and AlkPase, in addition to P_i uptake (26, 28), whereas recessive mutations in *PHO80* and *PHO85* result in their constitutive expression. *PHO2* mutants lack only the repressible APase (26, 28). *PHO6* and *PHO7* affect expression of *PHO3*, the "constitutive" APase (23), and *PHO9* encodes a protease involved in AlkPase maturation (9).

On the basis of genetic analysis of various double mutants and the discovery of a *cis*-dominant constitutive mutant (*PHO82*) contiguous to *PHO4* (26), Toh-e et al. originally proposed a role for the phosphatase regulatory genes involving transcriptional regulation via the sequential functioning of their products (28), reminiscent of control of phage development by repressor and antirepressor proteins. *PHO4* was considered to be a positive regulator essential for transcription of *PHO5* and *PHO8*. The *PHO80* and *PHO85* proteins form a repressor under negative control of *PHO81*. In the presence of P_i, this repressor blocks transcription of *PHO4* by binding to an adjacent site *pho82*. In the absence of P_i it dissociates, allowing for synthesis of *PHO4* which, in turn, associates with sites adjacent to *PHO5* and *PHO8* and activates transcription. For APase, this occurs in concert with *PHO2* at the *pho83* locus adjacent to *PHO5*.

More recent genetic studies, however, are inconsistent with this model. First, fine-structure meiotic mapping situates the *pho82* site in a narrow region within the *PHO4* locus (22). The existence of temperature-sensitive and nonsense suppressible mutations in *PHO4* argues strongly that this locus encodes a protein (22). The fact that two *PHO4* sites flank *pho82* therefore argues against a model defining the *pho82* site as an operator of *PHO4*. Moreover, APase activity shown by *PHO82 PHO4/pho82* (wild-type) *pho4* diploids grown under repressed conditions varied depending on the combination of *PHO82* and *pho4* alleles, unlike the *PHO82* homozygous diploids (22).

These findings led Toh-e et al. to propose a new model wherein the regulatory factors function simultaneously by direct molecular interaction (22). In its simplest form, this new model states that a few molecules of the regulatory factors are produced constitutively. During repression, the *PHO4* protein aggregates with a complex of *PHO80* and *PHO85* at a protein domain identified by *pho82* and is rendered unable to activate transcription of *PHO5*. Under derepressed conditions, the *PHO81* product binds to the *PHO80*, *PHO85*, *PHO4* aggregate, and the *PHO4* product is released. It binds, with the *PHO2* protein, to an operator of the structural gene and activates transcription.

Considerable biochemical and molecular evidence (5, 6, 11, 17) indicates transcriptional regulation of APase and AlkPase. Three distinct APase polypeptides (p60, p58, and

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TABLE 1. Genes and their proposed function in the phosphatase system

Proposed function (9,13,22–29)	Gene[a]	Chromosome location
Structural genes		
Constitutive APase; 57,000 daltons	*PHO3*[b] (*PHOC*)	II
Repressible APase I; 60,000 daltons	*PHO5* (*PHOE*)	II
Repressible APase II; 58,000 daltons	*PHO10*	
Repressible APase III; 56,000 daltons	*PHO11*	
Repressible AlkPase	*PHO8* (*PHOH*)	IV
P_i transport (permease)	*PHO84* (*PHOT*)	
Genes affecting APase and AlkPase expression		
(i) Concerted for P_i repression: pleiotrophic function		
Positive control	*PHO4* (*PHOD*)	VI
Negative control	*PHO80*[c] (*PHOR*)	XV
Negative control	*PHO85* (*PHOU*)	XVI
Mediator of *PHO80*/*PHO85* activity	*PHO81* (*PHOS*)	
(ii) Specific		
Positive factor for repressible APase expression	*PHO2* (*PHOB*)	IV
Maturation of repressible AlkPase	*PHO9*[d] (*PHOI*)	XVI
Positive factor for constitutive APase expression	*PHO6* (*PHOF*)	
Positive factor for constitutive APase expression	*PHO7* (*PHOG*)	
Regulatory sites		
Domain within *PHO4* defining negative interaction with *PHO80* or *PHO85* or both	*pho82* (*phoO*)	VI
cis-Dominant site contiguous to *PHO5*	*pho83* (*phoP*)	II

[a] Wild-type designations (22 [renamed]).
[b] The designation constitutive for *PHO3* is qualified because its expression is significantly lowered when *PHO5* is derepressed (17).
[c] Allelic with *TUP7*.
[d] Allelic with *PEP4*.

p56) encoded by three repressible APase mRNAs are the products of the *PHO5*, *PHO10*, and *PHO11* genes, respectively (5, 6). Derepression results from a cellular increase in all three mRNAs and subsequent de novo enzyme synthesis. The transcript for the constitutive APase (p57 mRNA) is encoded by the *PHO3* locus, adjacent to *PHO5*.

In this work we investigated the role of regulatory genes affecting expression of this multienzyme family. Our results confirmed that the action of these genes occurs before production of APase mRNA and that all of their functions are indispensible for normal regulation of mRNA synthesis. Further biochemical data show that the interplay among these genes is at their posttranslational level. We showed specifically that *PHO4* is not regulated transcriptionally by *PHO80* but rather is expressed constitutively, supporting the notion that the *PHO4* protein functions simultaneously with the other regulatory factors to control APase transcription. Feedback and autoregulatory control mechanisms responsible for fine-tuning this regulation of APase mRNA (6), however, are dependent on de novo protein synthesis.

MATERIALS AND METHODS

Yeast strains and media. The haploid yeast strains used in this work (Table 2) were generously provided by Akio Toh-e and Yasuji Oshima. H42 (ATCC 26922) has the wild-type genotype for the production of repressible APase and AlkPase as well as constitutive APase. All other strains were derived by mutagenesis of H42 or its derivatives and are meiotic segregants containing the mutant allele *pho3-1* to eliminate the constitutive APase. Supplemented minimal dextrose (SMD) medium containing 1.5 of either KH_2PO_4 (high P_i) or KCl (low P_i) per liter was prepared as described previously (5, 6).

Cell growth and nucleic acid extraction. Yeast cultures were started with fresh overnight inocula (approx. 3×10^8 cells per ml in SMD high-P_i medium) by dilution to an initial cell density of 10^6 per ml in either low- or high-P_i SMD medium. Low-P_i SMD cultures were supplemented with fresh high-P_i SMD medium so that the total amount of high-P_i SMD medium was 1/200 of the total culture volume. Cells were grown at 30°C to a density of 2.5×10^7 per ml, arrested by addition of cycloheximide (0.1 mg/ml), and immediately frozen on dry ice. Cultures for derepression or repression time course experiments were pregrown at 30°C to a density of 2×10^7 cells per ml and harvested by filtration. Previous experiments confirmed that filtration of cells does not cause a physiological perturbation (6). Cells were then rapidly suspended at the same cell density in appropriate fresh medium preequilibrated at 30°C. Samples were taken at various times and either frozen for later enzyme assay or rapidly filtered and frozen for preparation of RNA.

Cycloheximide was added to a final concentration of 0.1 mg/ml to inhibit further protein synthesis 5 min before repression, derepression, or temperature shift. The rate of protein synthesis and its inhibition by cycloheximide were determined by measurement of [^{35}S]methionine (800 Ci/mmol; Amersham Corp.) incorporation into trichloroacetic-acid-precipitable protein in cultures labeled at an initial concentration of 10 μCi/ml. Temperature shift experiments with conditional regulatory mutants were handled as described above except that cultures were grown at 25°C for

TABLE 2. Genotype and phenotype of APase mutant strains of *S. cerevisiae*

Strain	Genotype[a]	Repressible APase phenotype
H42	**a** *gal4 PHO3*	Wild type
P28-24C	**a** *pho3-1*	Wild type
P142-4A	α *arg pho5-1*	Recessive negative
YAT40	α *arg pho5-2*	Recessive negative
YAT130	**a** *arg PHO83*	Dominant constitutive
P143-4B	α *arg pho2*	Recessive negative
P144-2D	α *arg pho4*	Recessive negative
P145-2B	α *arg pho81*	Recessive negative
P146-8B	α *arg pho80*	Recessive constitutive
P188-1A	α *arg pho84*	Recessive constitutive
P189-1A	α *his pho85*	Recessive constitutive
R6-3A	α *pho4*(Ts)	Nonpermissive at 37°C
034-M69	**a** *pho80*(Ts)	Nonpermissive at 37°C
P1235-2A	**a** *pho2*(Ts)	Nonpermissive at 37°C

[a] All strains except wild-type H42 contain the *pho3-1* mutant allele to eliminate the constitutive APase.

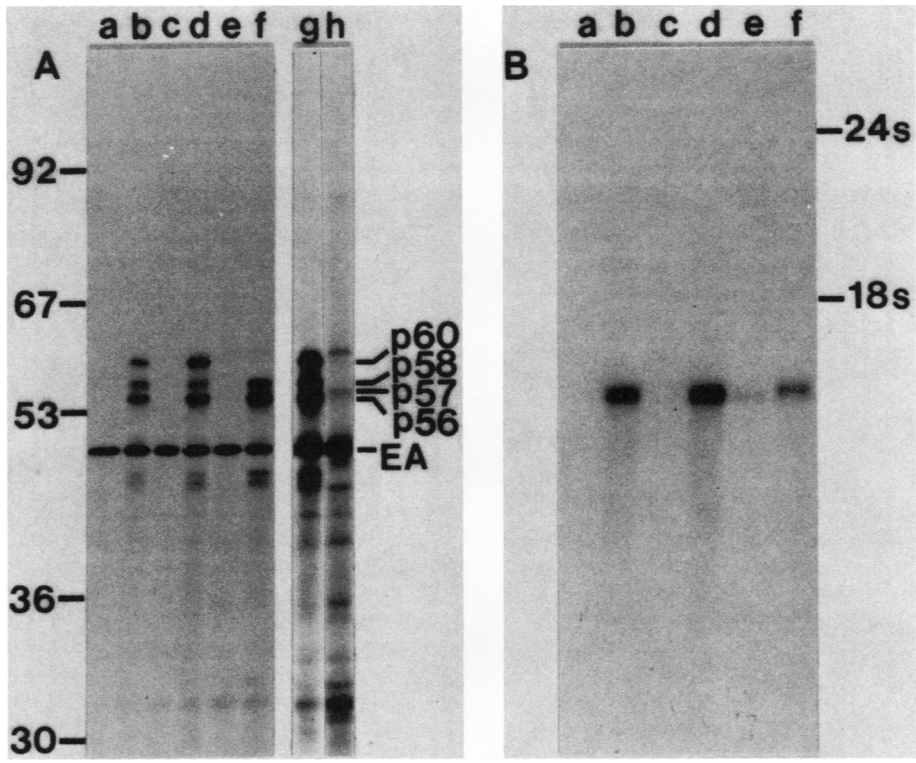

FIG. 1. Measurement of distinct APase mRNAs and RNA transcripts in wild-type and *pho3* and *pho5* mutant strains. (A) Translational analysis. RNAs prepared from various strains were translated in a wheat germ system with [^{35}S]methionine, and the radiolabeled translation products were immunoprecipitated with a mixture of APase and enolase immunoglobulin G. Immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Shown are the immunoprecipitated translation products of RNA from cells of strains H42 (a, b), P28-24C (c, d), and P142-4A (e, f) grown in high- and low-P_i SMD media, respectively. Lanes g and h are the same lanes as b and a, respectively, at a darker autoradiographic exposure to enable visualization of the *PHO3* product p57. (B) RNA blot hybridization analysis. Total yeast nucleic acid was electrophoresed on a 1.5% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pAP20 DNA (*PHO5*, *PHO3* probe). The RNAs analyzed (a to f) were from the same preparations as in panel A (a to f), respectively.

the permissive temperature and at 37°C for the nonpermissive temperature.

Nucleic acids were prepared from cells by standard H_2O-saturated phenol extraction in the presence of 0.1% (wt/vol) sodium dodecyl sulfate after cell disruption by mechanical breakage with glass beads (6).

APase enzyme activity was determined spectrophotometrically by using intact cells and *p*-nitrophenyl phosphate as substrate (5). One unit of enzyme activity was taken as that which liberated 1 μmol of *p*-nitrophenol per min. Cell density was measured by optical density at 660 nm (OD_{660}), and data were expressed in enzyme units per OD_{660} of cells.

TABLE 3. Enzyme and mRNA levels of cells grown in high- and low-P_i media

Strain	Enzyme activity (EU/OD_{660}) × 10^2	In vitro mRNA activity (relative density units) × 10^3				Hybridized RNA (relative density units) × 10^3
	High-P_i/low-P_i	High-P_i/low-P_i				High-P_i/low-P_i
		p60	p58	p57	p56	
H42 (*PHO5 PHO3*)	1.5/19.6	<1/105	<1/100	3/U[a]	<1/135	22/360
P28-24C (*PHO5 pho3*)	0.6/24.4	<1/160	<1/105	3/U	<1/160	22/450
P142-4A (*pho5 pho3*)	0.6/11.7	<1/<1	<1/105	14/U	<1/175	46/125
YAT40 (*pho5 pho3*)	0.6/6.0	<1/205[b]	<1/100	4/U	<1/175	
P143-4B (*pho2 pho3*)	0.6/2.0	<1/<1	<1/<1	10/7	<1/<1	27/14
P144-2D (*pho4 pho3*)	0.6/2.0	<1/<1	<1/<1	4/4	<1/<1	14/19
P145-2B (*pho81 pho3*)	0.9/1.9	<1/<1	<1/<1	24/4	<1/<1	50/20
YAT130 (*PHO83 pho3*)	4.3/22.7	23/45	<1/115	<1/U	<1/90	80/250
P146-8B (*pho80 pho3*)	12.0/38.7	26/150	9/92	U/U	21/150	85/400
P189-1A (*pho85 pho3*)	1.9/30.6	8/160	3/120	U/U	10/165	30/440
P188-1A (*pho84 pho3*)	6.3/50.6	15/—	6/—	U/—	19/—	58/—

[a] U, Unable to resolve band.
[b] Nonsense fragment.

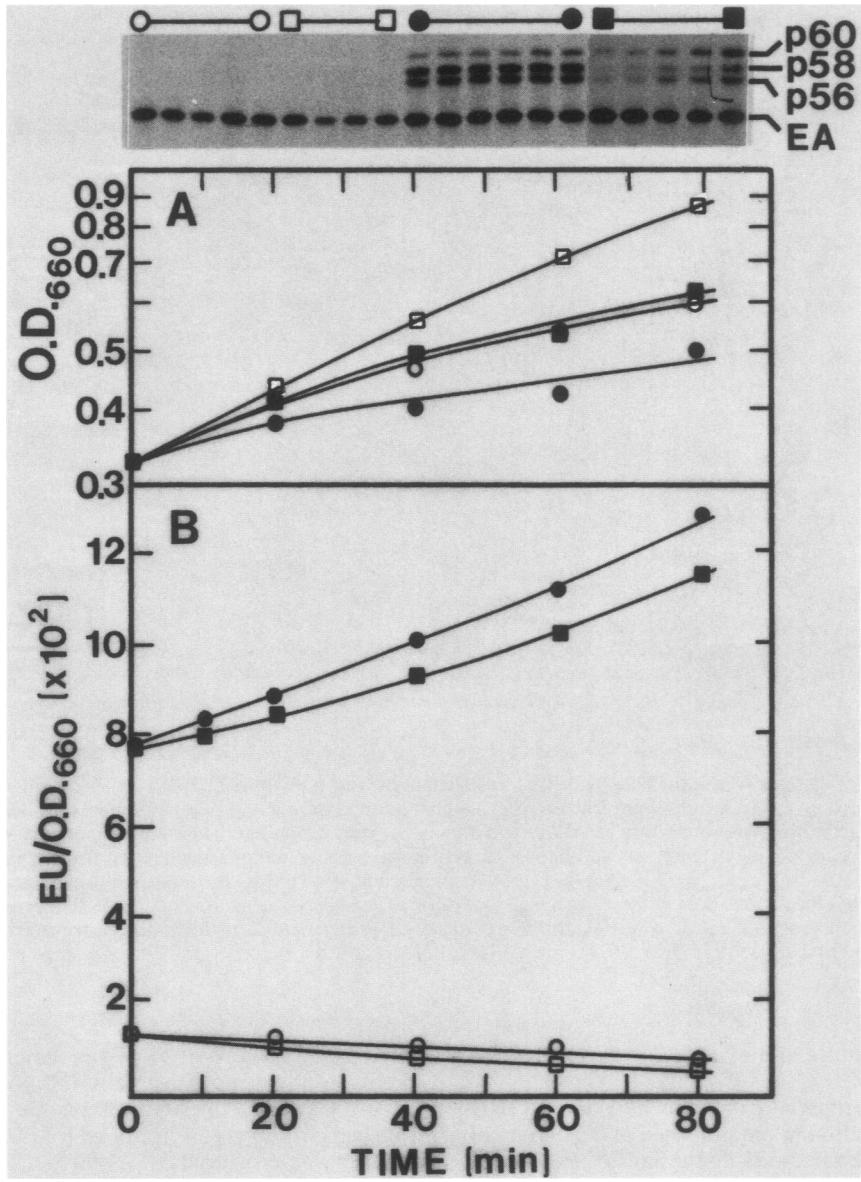

FIG. 2. Analysis of enzyme and mRNA levels in a *PHO5* strain after temperature shift. Cells of strain P28-24C were grown in low-P_i (●) or high-P_i (○) SMD medium at 25°C, and at an OD_{660} of 0.32 (*t*, 0 min) portions of each culture—low-P_i (■) and high-P_i (□)—were rapidly shifted to 37°C and incubated for 80 min. At intervals, samples were removed and assayed for (A) growth and (B) enzyme activity (EU, unit of enzyme activity). Gel autoradiogram: in vitro APase mRNA activity and abbreviations were as described in the legend to Fig. 1.

Analysis of in vitro translation products of total yeast RNA. Translations were performed in a wheat germ system by using [^{35}S]methionine (>800 Ci/mmol; Amersham) under conditions wherein radioisotope incorporation was linear with RNA concentration (6). Specific cell-free translation products were analyzed by immunoprecipitation, electrophoresis, and fluorography by published methods (6). Anti-enolase immunoglobulin G was added to reactions as an internal control. Densitometry was done with a Joyce-Loebl scanning densitometer (6).

RNA gel electrophoresis, nitrocellulose transfer, and hybridization. Total yeast nucleic acids were electrophoresed in 1.5% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized as described previously (6). Hybridization was done with a nick-translated (16) pBR322 recombinant plasmid with an 8-kilobase *PHO5*/*PHO3* DNA insert (17). Autoradiograms were quantitated by densitometry. The linearity of the hybridization signal and the translational activity with respect to APase mRNA concentration under the standard conditions used here were previously shown to be proportional throughout the concentration range examined (6). However, there are two limitations to the use of pAP20 as a probe in the hybridization assay. First, in addition to the *PHO5* gene this plasmid contains *PHO3*. Because *PHO3* is not expressed under low-P_i conditions and only at low levels under high-P_i conditions (17), it contributes negligibly to the hybridization signal. More significantly, the probe weakly hybridizes to *PHO10* and *PHO11* RNAs, which comigrate with *PHO5* transcripts in this gel system. From previous calculations, however, we estimated

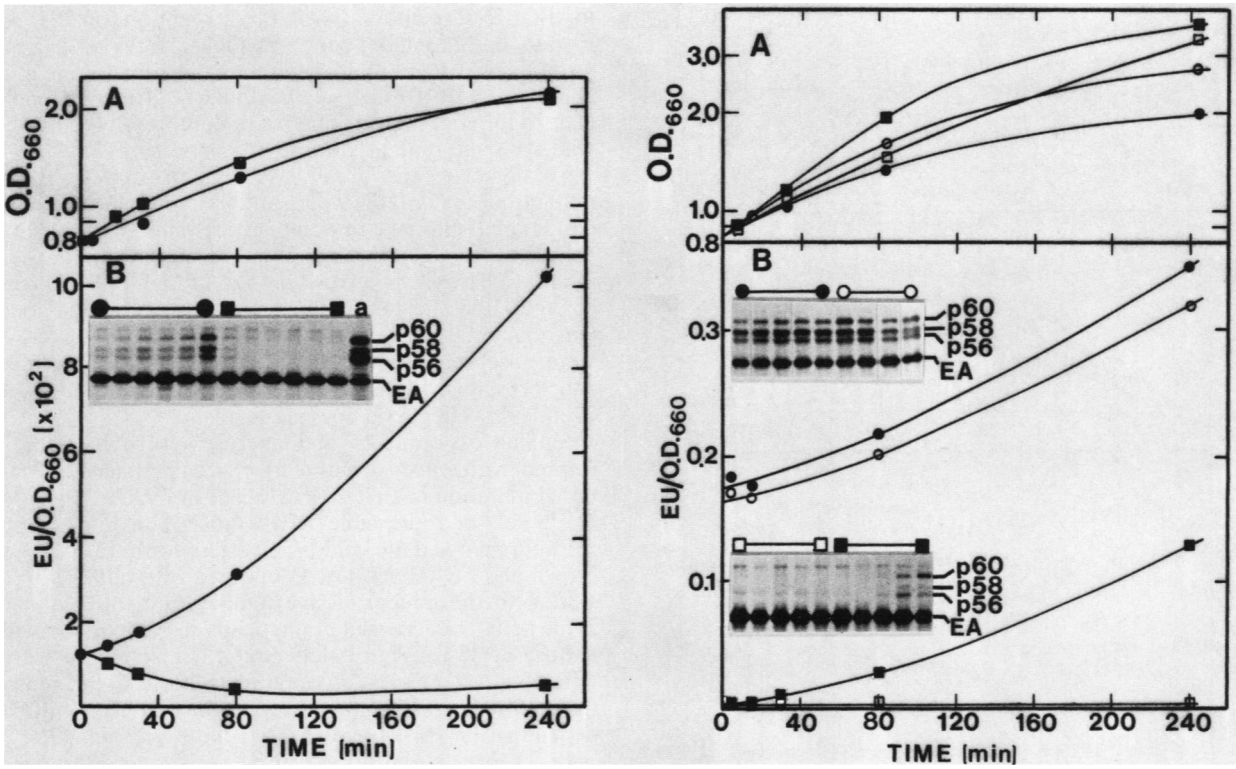

FIG. 3. Analysis of enzyme and mRNA levels in *pho4* and *pho80* conditional mutants after temperature shift. Left panel: Cells of strain R6-3A were grown in a low-P_i SMD medium at 25°C (●), and at an OD_{660} of 0.8 (*t*, 0 min) a portion of the culture (■) was rapidly shifted to 37°C. Both cultures were then incubated for 4 h and analyzed as described in the legend to Fig. 2. Lane a: Immunoprecipitated translation products of low-P_i RNA from cells of strain P28-24C about 50% derepressed for APase. Right panel: Cells of strain 034-M69 were grown in low-P_i SMD medium (●) or high-P_i SMD medium (□) at 25°C, and at an OD_{660} of 0.8 (*t*, 0 min) portions of each culture—low-P_i (○) and high-P_i (■)—were rapidly shifted to 37°C and analyzed as described in the legend to Fig. 2. Upper insert, low-P_i cultures; lower insert, high-P_i cultures.

that at least 85 to 90% of the hybridization signal from this probe is due to reaction with *PHO5* transcripts, enabling pAP20 to be useful in this assay (6).

RESULTS

***PHO2*, *PHO4*, and *PHO81* are essential for transcription; *pho80* and *pho85* result in constitutive expression.** We previously developed two assays to measure variations in levels of repressible APase mRNAs in response to changes in extracellular concentration of P_i (5). Cells were grown under repressed (high-P_i SMD medium) or derepressed (low-P_i SMD medium) conditions, and RNA was isolated and either translated in an in vitro wheat germ system or analyzed by RNA blot hybridization. The translation assay discriminated among three distinct APase mRNAs, based on the products they encode (p60, p58, p56) (Fig. 1A), whereas the hybridization assay semiquantitatively measured the concentration of *PHO5* transcript (Fig. 1B).

These assays were used to determine whether any of the genes known to affect APase activity act at the level of transcription and whether they control all three repressible APase genes coordinately. Strains carrying a mutation in the constitutive APase structural gene (*pho3-1* allele) and one of the regulatory loci were analyzed for enzyme and mRNA activity. The results are summarized in Table 3. Only *PHO5* mRNA activity was affected by mutations in strains P142-2A (*pho5-1*) (Fig. 1), YAT40 (*pho5-2*), and YAT130 (*PHO83*). The *pho5-1* strain failed to produce mRNA detectable by the in vitro translation analysis and yielded considerably reduced levels of hybridizable RNA, probably representing the amount of crosshybridization of the probe with *PHO10* and *PHO11* transcripts. The *pho5-2* strain produced a p60 product upon in vitro translation; the low levels of enzyme activity suggest that this allele encodes an inactive or labile enzyme. The *PHO83* strain expressed *PHO5* constitutively but at lower levels than the wild-type, low-P_i-grown cells. This phenotype is owing to the insertion of a transposable element into the *PHO5* promoter (25).

Repressible APase mRNAs (p60, p58, p56) were undetectable under repressed or derepressed conditions in cells of strain P143-4D, P144-2D, or P145-2B, which carry mutations at *PHO2*, *PHO4*, and *PHO81*, respectively. Levels of enzyme activity were equivalent to the basal level of activity of P28-24C (*pho3-1*, wild type for repressible APase) grown in high-P_i medium. Levels of *pho3-1* RNA in the *pho4* and *pho81* strains were elevated two- to threefold over the corresponding level in P28-24C.

In contrast, repressible APase mRNA was present constitutively in strains P146-8B and P189-1A (*pho80* and *pho85*, respectively) and P188-1A (*pho84*) (data not shown). Repressible APase mRNA levels for derepressed cells were similar to the *PHO5 PHO10 PHO11* control, P28-24C. In high-P_i medium, levels of repressible APase mRNAs were 5 to 15% of the level observed in low-P_i medium.

All three repressible APase mRNAs were regulated in concert, and their ratios were relatively constant within these congenic strains. Hybridizable RNA levels paralleled translatable mRNA levels, and enzyme activity reflected

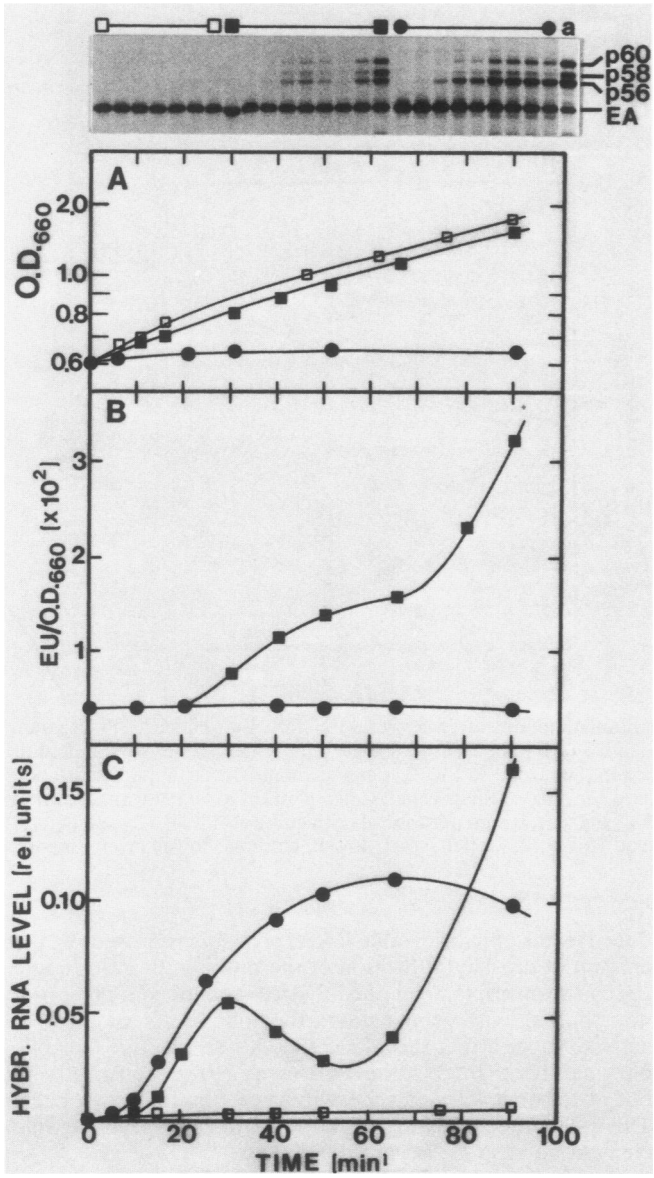

FIG. 4. APase derepression after inhibition of protein synthesis by cycloheximide. Cells of strain P28-24C were grown in high-P_i SMD medium to an OD_{660} of 0.6 (t, 0 min) and divided into three portions. Cycloheximide was added to one portion to a final concentration of 100 g/ml. After 5 min of incubation, cells from all three portions were harvested by membrane filtration and washed. The cycloheximide-treated portion of cells was suspended in low-P_i SMD medium containing cycloheximide (●). One of the remaining two portions (untreated) was suspended in low-P_i SMD medium (■), whereas the other was returned to high-P_i SMD medium (□). All three cultures were then further incubated for 1.5 h. At intervals, samples were removed and assayed for (A) growth, (B) enzyme activity (EU, unit of enzyme activity), and (C) hybridizable APase RNA, as described in the legend to Fig. 1. The relative intensities of RNA bands in the RNA blot autoradiogram are expressed in relative density units as a ratio to the amount of hybridizable RNA from a fully derepressed SMD culture of strain P28-24C. Gel autoradiogram: in vitro APase mRNA activity. Lane a: Control as described in the legend to Fig. 3.

total mRNA activity (with the exception of the *pho5-2* mutant discussed above). The higher ratio of enzyme to mRNA activity in low-P_i cultures of the constitutive mutants compared with P28-24C cells similarly grown probably reflects differences in the kinetics of derepression between the wild-type and mutant strains.

To correlate the above assignments directly with the mutant alleles cited, we examined temperature-sensitive conditional mutants in temperature shift experiments. A control experiment was first performed with a wild-type strain. Cells were pregrown in low- or high-P_i SMD medium at 25°C, and the temperature was rapidly raised to 37°C. Only marginal and temporary effects on APase mRNA levels were observed (Fig. 2), presumably in relation to the heat shock response of yeast cells (14). The temperature shift had even lesser effects on enzyme levels.

Similar experiments and analyses were therefore performed with *pho3* strains carrying a heat-sensitive conditional mutation in *PHO2* (P1235-2A) or *PHO4* (R6-3A) (Fig. 3). Levels of repressible APase mRNA in low- or high-P_i medium followed the wild-type pattern for both strains when grown at 25°C. When low-P_i cultures of strain R6-3A were shifted to the nonpermissive temperature, mRNA and enzyme activity decreased dramatically in comparison with the culture at 25°C (Fig. 3, left panel). Similar data were obtained with the *pho2*(Ts) strain (data not shown), indicating that normal, coordinate derepression of APase mRNAs is dependent on *PHO4* and *PHO2* gene expression.

In a separate experiment (data not shown), the *pho4*(Ts) strain was analyzed by pregrowing cells under derepressed conditions but at the nonpermissive temperature and then shifting to 25°C. Neither enzyme or mRNA was detected at 37°C, but derepression of both occurred coincident with the temperature shift.

Temperature shift experiments were also performed on a *pho3* strain that carries a heat-sensitive conditional mutation in *PHO80* (O34-M69). Cells were grown in low- or high-P_i medium at 25°C and were rapidly shifted to 37°. Enzyme and mRNA levels in the two low-P_i cultures followed the wild-type pattern. In the high-P_i culture at 25°C, no mRNA was observed. Shifting the culture to the nonpermissive temperature, however, led to derepression of mRNA and enzyme (Fig. 3, right panel), indicating that *PHO80* function is necessary for repression of *PHO5*, *PHO10*, and *PHO11* transcription under high-P_i conditions.

De novo protein synthesis is not prerequisite for derepression or repression of APase. To determine whether de novo protein synthesis is required for derepression of *PHO5*, *PHO10*, and *PHO11* transcripts, we performed cycloheximide inhibition experiments. The addition of cycloheximide at 100 μg/ml to growing cultures completely and immediately (within 2 min) halted [^{35}S]methionine incorporation into trichloroacetic-acid-precipitable protein but had little immediate effect on mRNA synthesis (data not shown). Cells of strain P28-24C were pregrown in high-P_i SMD medium under repressed conditions, cycloheximide was added, and incubation continued for 10 min. Cells were harvested and suspended in low-P_i SMD medium supplemented with cycloheximide. As anticipated, enzyme derepression did not occur in the presence of cycloheximide (Fig. 4). In contrast, APase mRNAs accumulated in its absence or presence. The control culture showed a typical biphasic appearance of mRNA, oscillating over hour 1 of derepression. The culture with cycloheximide showed an early and continuous rate of transcript accumulation before secondary effects of cycloheximide limited overall transcription (19). Thus, de novo

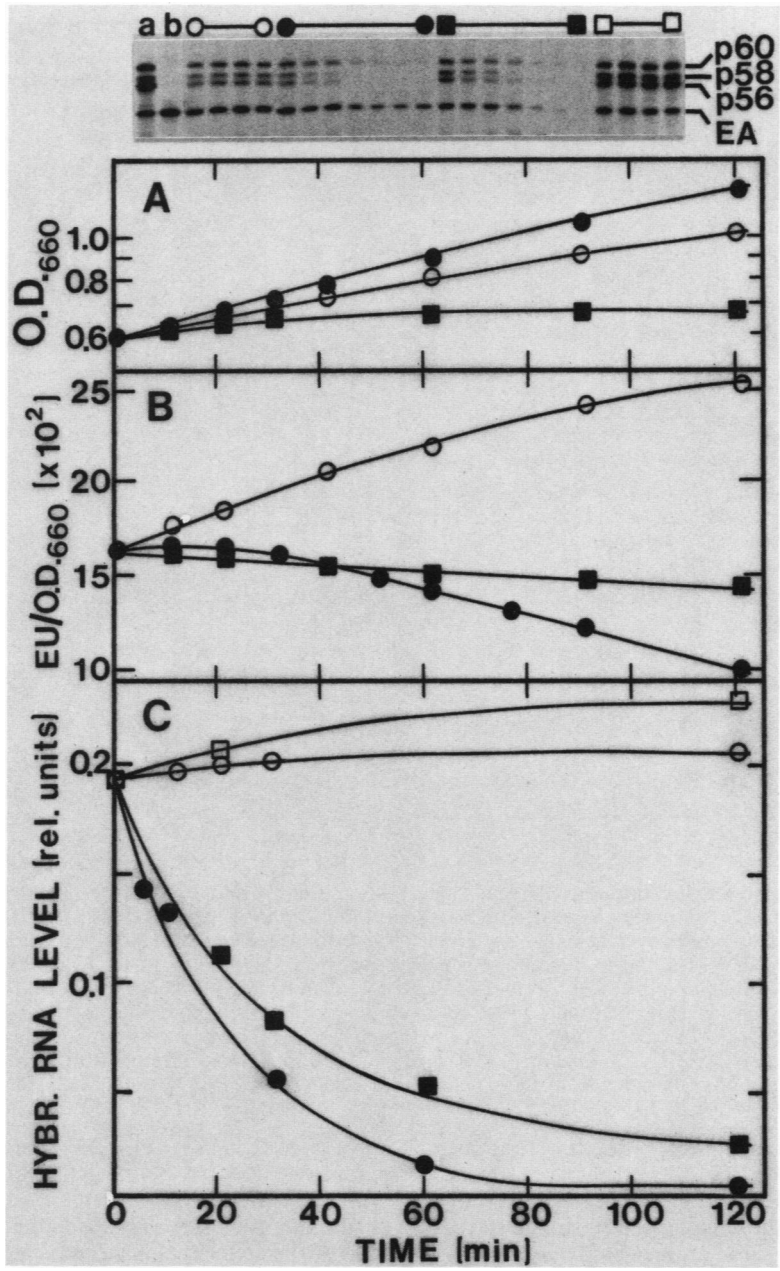

FIG. 5. APase repression after inhibition of protein synthesis by cycloheximide. Cells of strain P28-24C were grown in low-P_i SMD medium to an OD_{660} of 0.6 (*t*, 0 min) and divided into four portions. One portion was unchanged (○), one portion was supplemented with cycloheximide (□), one portion was repressed by the addition of P_i (●), and one portion was repressed by P_i 5 min after supplementation with cycloheximide (■). All four cultures were then further incubated for 2 h and analyzed as described in the legend to Fig. 4. Lane a: Same as lane a, Fig. 3. Lane b: Immunoprecipitated translation porducts of RNA from fully repressed cells of strain P28-24C.

protein synthesis is not required for activating APase transcription but is necessary for the feedback and autoregulatory repression responsible for early mRNA oscillations.

A similar experiment was performed to assess the constitutivity of the factors involved in repression of APase. Cells of strain P28-24C were pregrown under derepressed conditions (low-P_i SMD medium), cycloheximide was added, and this was followed 5 min later by the addition of P_i. A similar pattern of repression occurred in the presence or absence of cycloheximide (Fig. 5).

The repression observed for cycloheximide-treated cells may reflect either the normal repression of APase upon P_i addition or turnover of unstable factors required to maintain APase transcription. Their stability was therefore examined in cells exposed to cycloheximide for several hours. Cells of strain P28-24C were grown to a midpoint in APase derepression and treated with cycloheximide. Their ability to continue transcription of APase mRNA was then determined. RNA levels were compared with those from portions of the culture left to undergo normal derepression or repressed by the addition of P_i (Fig. 6).

The culture that was allowed to undergo full derepression progressed through transient feedback and autoregulatory repression to high APase RNA and enzyme levels. The culture supplemented with P_i midway through derepression underwent a rapid reduction in the level of APase RNA,

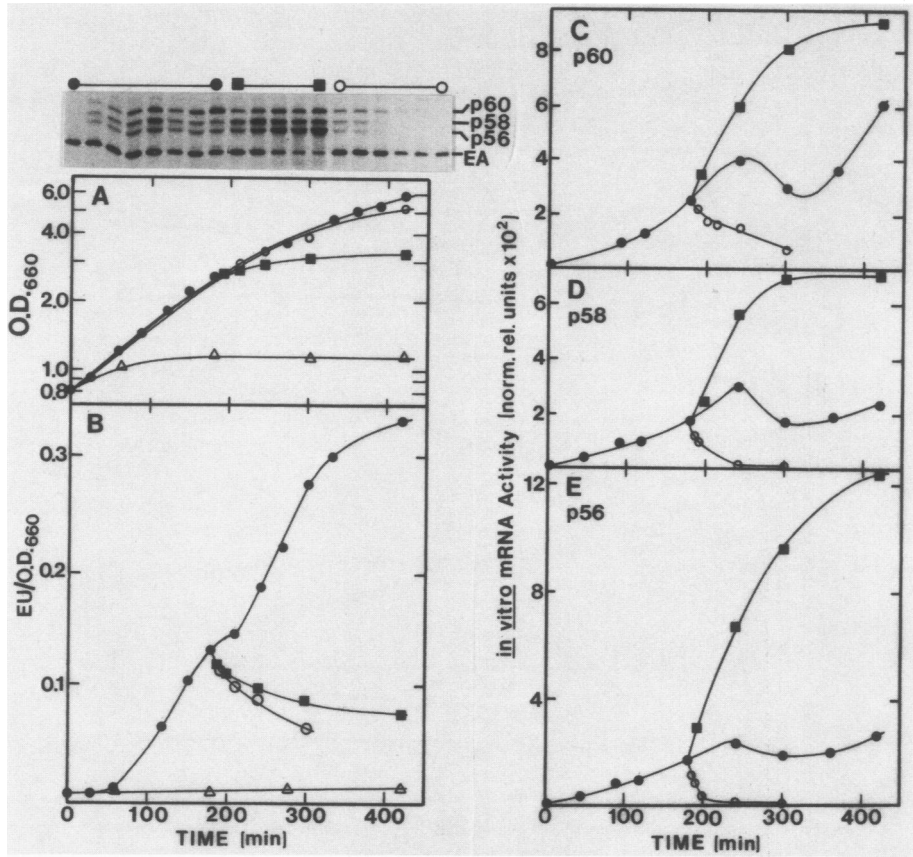

FIG. 6. Analysis of APase derepression during continuous exposure to cycloheximide. Cells of strain P28-24C grown at 30°C in high-P_i SMD medium to an OD_{660} of 0.6 (t, 0 min) were harvested by membrane filtration, washed, suspended in low-P_i SMD medium, and divided into two portions: with (△) or without (●) cycloheximide. After 3 h of incubation, the low-P_i SMD culture without cycloheximide, partially derepressed for APase, was further divided: one portion was unchanged (●), one portion was inhibited by addition of cycloheximide (■), and one portion was repressed by the addition of P_i (○). All four cultures were then further incubated for 4 h and analyzed as described in the legend to Fig. 3. The relative intensities of protein bands in the gel autoradiogram (panel C, p60; panel D, p58; panel E, p56) were determined by scanning densitometry and expressed in relative units as a ratio to the maximal level of APase mRNA activity (total) for RNA isolated from a fully derepressed culture of strain P28-24C grown in low-P_i SMD medium. Data are normalized to the corresponding yield of enolase mRNA, which remains a constant proportion of the total RNA during changes in cellular phosphate concentration (6).

presumably reflecting an immediate cessation of transcription and rapid mRNA turnover (6). A much slower reduction was observed for enzyme levels, consistent with its known stability. In contrast, the culture with cycloheximide showed a continuous increase in all three repressible APase mRNAs throughout the experiment, accompanied by a slow reduction in enzyme level. Transient feedback and autoregulatory inhibition of mRNA accumulation did not occur, resulting in mRNA derepression kinetics exceeding those of the normally derepressed control culture. Because APase mRNA was only slightly more stable in repressed cell cultures incubated with cycloheximide than without (Fig. 5), the ability to proceed from a midpoint in derepression in the presence of cycloheximide indicated that any positive regulatory factors required for transcription of *PHO5*, *PHO10*, and *PHO11* were stable for at least the duration of this experiment. We conclude, therefore, that de novo protein synthesis is unnecessary for repression of APase transcription.

***PHO80* does not regulate transcription of *PHO4*.** To test whether *PHO80* and *PHO85* regulate transcription of *PHO4*, as proposed elsewhere (28), we used the cycloheximide inhibition strategy to analyze high-P_i expression of APase in a *pho80*(Ts) mutant. Strain 034-M69 [*pho80*(Ts) *pho3*] was pregrown in high-P_i SMD medium at the permissive temperature (no enzyme synthesis). Portions of this culture were rapidly shifted to 37°C in the presence or absence of cycloheximide. As in the experiment of Fig. 3, the shift to 37°C in the absence of cycloheximide resulted in derepression of APase mRNA and enzyme activity. In the presence of cycloheximide, enzyme derepression did not occur upon temperature shift (Fig. 7), consistent with the known inhibition of protein synthesis; APase mRNA, however, underwent derepression to levels similar to those of the culture lacking cycloheximide. Because the accumulation of enzyme is dependent on *PHO4*, even in the absence of *PHO80* (22, 28), sufficient levels of the *PHO4* gene product must have been present in the cycloheximide-inhibited culture before the temperature shift.

DISCUSSION

We previously showed that repression and derepression of yeast APase in response to changes in extracellular P_i operate by regulation of cellular levels of mRNA (5, 6). Several lines of evidence indicate that this occurs at the level of transcription rather than posttranscriptionally. None of our experiments or those by others (1, 5, 6, 17) revealed modified or precursor RNAs which might serve as points of

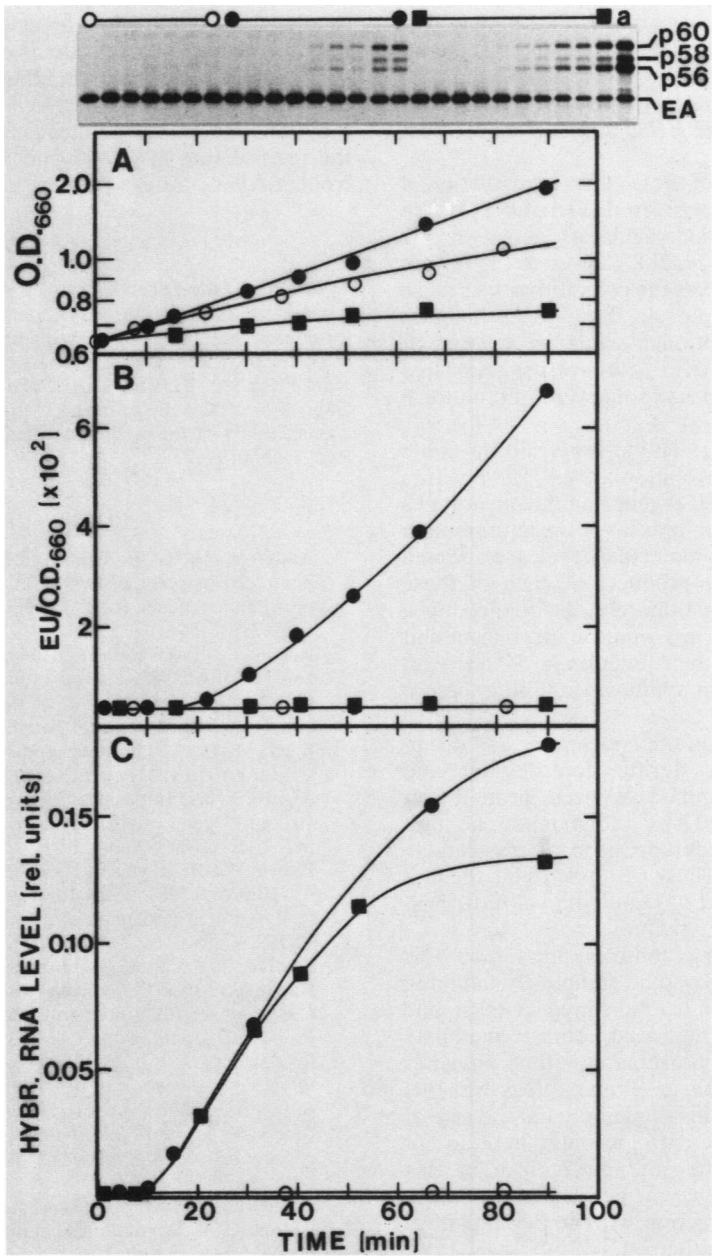

FIG. 7. APase derepression in a *pho80* conditional mutant upon temperature shift, after inhibition of protein synthesis by cycloheximide. Cells of strain 034-M69 were grown in high-P_i SMD medium at 25°C (permissive temperature) to an OD_{660} of 0.6 (*t*, 0 min). The culture was then divided into three portions: one portion remained unchanged (○), and two portions were rapidly shifted to 37°C, one without (●) and one with (■) cycloheximide added 5 min before the temperature shift. All three cultures were then further incubated for 1.5 h and analyzed as described in the legend to Fig. 4. Lane a: Same as lane a, Fig. 3.

regulation. Moreover, no known intervening sequences have been identified by nucleotide sequence analysis of the major APase gene *PHO5* (2, 4). The measured half-lives of *PHO5*, *PHO10*, and *PHO11* mRNAs in repressed cells are in agreement with the average half-life of stably expressed yeast mRNAs (3, 6). In vitro translation and RNA blot hybridization of total yeast RNAs show no evidence for nuclear accumulation of APase mRNAs in repressed cells. Finally, research in our laboratory and others (12, 15; D. Rogers, personal communication) has shown P_i-regulated expression of heterologous proteins in yeast cells resulting from hybrid gene fusions employing only the 5′ flanking promoter region of *PHO5*. We conclude from these data that control of *PHO5* expression is at the level of mRNA synthesis.

In this report we show the following. (i) The *PHO2*, *PHO4*, *PHO80*, *PHO85*, and *PHO81* genes all acted to regulate the level of repressible APase transcription. (ii) These genes exerted a strict, coordinate control on the three repressible APase structural genes. (iii) The regulatory factors encoded by these genes were expressed constitutively in repressed and derepressed cells. (iv) *PHO4* was not regulated transcriptionally by the products of *PHO80* and *PHO85*. (v) Feedback and autoregulatory controls fine-tuning the regulation of repressible APase were dependent on de novo synthesis of protein.

We began our investigation of the mechanism of action of the phosphatase regulatory genes by determining their effects on APase transcript levels. We showed that wild-type alleles of *PHO2*, *PHO4*, and *PHO81* are indispensible for mRNA accumulation, whereas *PHO80* and *PHO85* are necessary for mRNA repression.

Various models for the roles of these genes in regulation of APase transcription may be considered. Genetic evidence indicates that all of the identified regulatory genes encode diffusible protein products (22, 24, 28). Central to the formulation of such models, therefore, is the constitutivity of these regulatory proteins. For example, do these genes function through a cascade of transcriptional regulatory events, or does regulation occur by the direct action of the translated products of these regulatory genes in some type of functional network?

The two models for APase regulation proposed by Toh-e et al. (22, 28) represent these two alternatives. The position of the *pho82* locus within the *PHO4* gene and the phenotypes of heterozygous *pho82 PHO4* diploids favor the latter model. To test these hypotheses at the molecular level, we adopted the following argument. If the products of each of these genes are not synthesized constitutively, as implied by a transcriptional cascade model, we would expect to inhibit their production and therefore their function in APase regulation by the addition of protein synthesis inhibitors before derepression or repression.

The effect of cycloheximide on the appearance and disappearance of APase mRNAs during derepression and repression was therefore determined. Whereas protein synthesis was immediately inhibited by cycloheximide, no inhibition of either repression or derepression of translatable APase mRNAs was observed. These results provide the first biochemical support for a model of regulation via posttranslational interaction of regulatory factors.

Feedback and autoregulatory controls fine-tuning this regulation were, however, shown to be completely inhibited by the addition of cycloheximide, indicating at least one point at which translationally mediated controls modulate cellular levels of APase and presumably other enzymes involved in phophorus metabolism. Previous experiments showed a correlation between this regulation and the size of the polyphosphate pool (6). The data presented here do not enable us to determine whether the mechanism of this regulation is dependent on the enzyme activity of the APase protein or on its physical interaction with some regulatory factor before its transport from the cell.

Finally, we argued, if any component of the regulatory mechanism were inducible, as proposed for *PHO4* (28), then protein synthesis would be required to derepress APase in high-P_i cultures of a temperature-sensitive *pho80* mutant when shifted to a nonpermissive temperature. The accumulation of translatable APase mRNA upon shift in temperature in such experiments, however, was insensitive to cycloheximide inhibition. This derepression occurred during continuous incubation in the high-P_i medium, indicating that the entire machinery for transcriptional expression of the APase structural genes was present, in support of the above revised model (22) for a direct interplay of these regulatory gene products in the control of APase structural gene expression. Thus, although *PHO80* is hypostatic to *PHO4* (28), it does not regulate *PHO4* transcriptionally.

Analysis of RNA transcripts from the recently cloned *PHO4* gene (R. Koren and K. Bostian, submitted for publication) support this conclusion. Although the above model provides a view of how the APase structural gene might be regulated in reponse to transcriptional controls, it is as yet unknown whether the products of these regulatory genes act by direct intermolecular binding, by interaction with DNA regulatory sequences or DNA-binding proteins, or whether they function via a cytoplasmic role. Further work to define the precise role of *PHO4* and other regulatory genes controlling APase transcription is under way.

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