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Physiological Control of Repressible Acid Phosphatase Gene Transcripts in *Saccharomyces cerevisiae*

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We have examined the regulation of repressible acid phosphatase (APase; orthophosphoric-monoester phosphohydrolase [acid optimum], EC 3.1.3.2) in *Saccharomyces cerevisiae* at the physiological and molecular levels, through a series of repression and derepression experiments. We demonstrated that APase synthesis is tightly regulated throughout the growth phase and is influenced by exogenous and endogenous P_i pools. During growth in a nonlimiting P_i medium, APase is repressed. When external P_i becomes limiting, there is a biphasic appearance of APase mRNA and enzyme. Our data on APase mRNA half-lives and on the flux of intracellular P_i and polyphosphate during derepression are consistent with a mechanism of transcriptional autoregulation for the biphasic appearance of APase mRNA. Accordingly, preculture concentrations of P_i control the level of corepressor generated from intracellular polyphosphate degradation. When cells are fully derepressed, APase mRNA levels are constant, and the maximal linear accumulation rate of APase is observed. A scheme to integrate phosphorus metabolism and phosphatase regulation in *S. cerevisiae* is proposed.

Phosphorus metabolism in *Saccharomyces cerevisiae* involves five principal enzymes for the acquisition and metabolic integration of P_i. These enzymes regulate intracellular concentrations of P_i by a cyclic pathway of polyphosphate (polyP) synthesis and degradation (18, 47). The polyP is sequestered into vacuoles and forms the most abundant phosphate compounds in yeasts. The majority is an acid-insoluble polymer of 240,000 daltons, whereas the remainder consists of short-chain-length acid-soluble residues (42).

Both a phosphate permease (39) and acid phosphatase (APase) (41) participate in phosphorus acquisition. APase (orthophosphoric-monoester phosphohydrolase [acid optimum], EC 3.1.3.2) is an exocellular enzyme active on a broad spectrum of phosphoester substrates (41). Incorporation of P_i as a monovalent anion occurs by an energy-dependent permease transport mechanism (9, 39). Intracellular P_i is converted via ATP to polyP by the unique enzyme, polyP kinase, which transfers the terminal phosphoryl group of ATP to polyP. Degradation of polyP, on the other hand, is catalyzed by several enzymes, including polyP kinase. However, the direct interconversion of polyP to ATP by polyP kinase does not occur in vivo. Rather, polyP is sequentially hydrolyzed to smaller-chain-length molecules by polyphosphatase, and by the vacuolar enzyme, alkaline phosphatase (10, 22, 29).

When yeast cells are starved for phosphate, internal reserves of polyP are depleted (49), and overall cellular metabolism becomes dependent upon exocellular APase (41, 46). Although a low basal level of this enzyme is expressed when cells are grown in a P_i-rich medium, upon P_i starvation, the majority is derepressed, and APase may constitute as much as 1% of the total yeast protein. Concurrently, polyP kinase, polyphosphatase, and alkaline phosphatase levels are elevated. When exogenous P_i is replenished, cells enter a phase of rapid polyP synthesis (30), and both APase and alkaline phosphatase are repressed.

Little is known about the molecular or physiological mechanisms regulating the expression of these enzymes. Repression of APase and alkaline phosphatase has been shown previously (21, 45, 46) to be due to the action of a number of regulatory genes dispersed throughout the yeast genome. From this genetic data, a model was formulated wherein alkaline phosphatase and APase are transcriptionally regulated by a coordinate mechanism involving the products of numerous regulatory genes interacting at the level of their post-translational expression (7, 45). Biochemical support for this model was forthcoming with the characterization by Bostian et al. (8) of the functional expression of APase

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structural genes, and subsequently by Rogers et al. (38) after the isolation of these genes by Kramer and Andersen (24).

Repressible yeast APase is an exocellular glycoprotein consisting of several sequence-related polypeptides (D1 through D4) synthesized from three distinct mRNAs (p60, p58, and p56) (7). The p60 protein (60,000 daltons) has been shown to be the major APase polypeptide (D1) encoded by the gene *PHO5*, defined by genetic methods (7, 45, 46). A second APase gene, unlinked to *PHO5* (38), encodes p56 (56,000 daltons), the in vitro form of D4. Both genes have been cloned and characterized (24, 38). D2 and D3 are processed forms of the p58 polypeptide (58,000 daltons) and are encoded by a third APase gene that has remained uncharacterized. Gene products from all three genes are glycosylated, secreted, and catalytically active. A "constitutive" APase, encoded by the *PHO3* gene, is thought to be the 57,000-dalton product of a gene shown physically to be contiguous with *PHO5* (4).

In our investigation of the genetic regulation of the yeast phosphatase system, we have become interested in the role of exogenous and endogenous P_i reserves on the physiological control of APase synthesis. The ability to probe cellular RNAs for APase gene transcripts combined with the ability to measure mRNA activity in vitro enabled us to directly follow the regulation of transcription and translation of the APase genes. Experimental observations of transcript and enzyme levels are consistent with a mechanism of transcriptional autoregulation for expression of the APase genes. Measurements of endogenous and exogenous phosphorus pools and data on APase mRNA half-lives support this interpretation . Although a mechanism for autoregulation of *PHO5* mRNA has not been established, the data indicate that the metabolic balance of the polyP cycle is intimately involved.

MATERIALS AND METHODS

Yeast strains and media. The haploid *S. cerevisiae* strain P28-24C (**a** *pho3-1*) synthesizes both repressible APase and alkaline phosphatase but fails to produce the constitutive APase. This strain was derived from wild-type strain H42 (ATCC 26922). Both were kindly provided by Akio Toh-e, Osaka University, Osaka, Japan. The wild-type diploid, Y185 (ATCC 32748), was from our own stock collection. Burkholder minimal medium (pH 4.7) (46) contained 1.5 g of KCl (low P_i) or 1.5 g of KH_2PO_4 (high P_i) per liter. SMD medium (pH 4.7) consisted of Burkholder minimal medium supplemented with one-fifth strength low-P_i YEP (1% yeast extract, 2% Bacto-Peptone [Difco Laboratories]) (8). Low-P_i and high-P_i SMD medium also contained 1.5 g of KCl or KH_2PO_4 per liter, respectively.

Bacterial plasmids. The recombinant plasmid pAP20 contains the *PHO3* and *PHO5* genes within an 8-kilobase *Eco*RI yeast DNA fragment inserted at the *Eco*RI site of pBR322. These plasmids have been characterized elsewhere (38). The 8-kilobase *Eco*RI yeast DNA fragment was subcloned from λ YIp20 (24), kindly provided by Richard Kramer (Hoffman-LaRoche, Nutley, N.J.). The construction of pAP20 and the preparation of plasmid DNA were by standard techniques (40).

APase activity. APase activity was measured in whole cell suspensions or in culture supernatants by a spectrophotometric assay with *p*-nitrophenol phosphate as substrate, as previously described (7). One unit of enzyme activity is that amount of enzyme liberating 1 μmol of *p*-nitrophenol per min.

PolyP determination. Acid-soluble and acid-insoluble polyP were prepared from cells by the method of Katchman and Fetty (22), except that polyP was separated from nucleotides and nucleic acids by using activated charcoal (35) rather than by barium precipitation. Acid-insoluble polyP was determined after hydrolysis to P_i (22). Acid-soluble polyP, which includes PP_i, was the difference between the phosphate content of the acid-soluble fraction before and after hydrolysis to P_i. Intracellular P_i was the phosphate content before hydrolysis. Phosphate concentration was determined by the method of Fiske and Subbarow (14).

Extracellular P_i determination. P_i concentration in culture medium was determined by the method of Dulley (11), permitting assays of up to 0.1 μg of phosphorus per ml in the presence of detergent or protein.

Determination of cell number and cell size distribution. Culture densities were determined by counting cells in an electronic cell counter (Coulter counter, model B) equipped with a 50-μm aperture tube, after their dilution to approximately 5×10^4 cells per ml in saline. Median cell volume and size distribution were determined with a particle size distribution analyzer (Coulter Channelyzer, model C-1000) equipped with an X-Y plotter. Median cell volume was calculated from these data by the method of Koch and Blumberg (23). Volume calibrations were made with 4.96-μm-diameter (72 μm^3) organic beads (Coulter). The percentage of budded cells in these cell populations was determined by microscopic examination of 100 to 200 cells.

Purification of APase. Repressible APase was purified to homogeneity by the method of Boer and Steyn-Parve (4) with minor modifications, as previously described (8). Partially purified intracellular and extracellular APase were prepared from cells of strain H42, steady-state labeled with [^{35}S]methionine (40 μCi/ml) in low-P_i Burkholder minimal medium supplemented with 0.05× low-P_i YEP. Extracellular APase was prepared from the culture supernatant by ethanol precipitation after removal of the cells by membrane filtration. Intracellular APase was prepared from washed cells by the cell fractionation and low-temperature precipitation steps of the standard purification procedure and finally concentrated by ethanol precipitation. All three preparations were deglycosylated with endoglycosidase H as previously described (8).

Cell growth and RNA isolation. The cell cultures used in these experiments were grown at 30°C with good aeration and were started with fresh overnight inocula (at approximately 3×10^8 cells per ml) by dilution to an initial cell density of 5×10^6 cells per ml.

Growth measurements were made by following the optical density of the culture at 660 nm (OD_{660}). Samples were taken at various times for the determination of enzyme activity or for the preparation of RNA. Cell samples for RNA isolation were filtered rapidly onto 0.45-μm HAWP Millipore filters and immediately frozen on dry ice. Total cellular RNAs were then extracted by standard water-saturated phenol extraction in the presence of 0.1% (wt/vol) sodium dodecyl sulfate after cell disruption by mechanical breakage with glass beads (6, 19). In some experiments, cells were shifted from high- to low-P_i growth medium. In these cases, cultures were allowed to reach an OD_{660} of 0.6, and then cells were rapidly collected by membrane filtration. Filtered cells were washed with suspension medium and then suspended at the same density into medium pre-equilibrated at 30°C. Rates of radioisotope incorporation into protein and protein-specific activities were determined by trichloroacetic acid precipitation (19) and by the protein determination of Lowry et al. (31). RNA was quantitated by the method of Ogur and Rosen (36).

Cell-free protein synthesis. Total yeast cellular RNAs were translated in a wheat germ cell-free system with [^{35}S]methionine (>800 Ci/mmol; Amersham) by previously published methods (6, 8). All translations were performed under standard conditions where radioisotope incorporation was linear with RNA concentration. Translation reaction products were analyzed by immunoprecipitation, electrophoresis, and fluorography as described previously (6, 8). The mRNA activities for the synthesis of corresponding immunoprecipitated proteins were determined by densitometry of gel autoradiograms, using a Joyce-Loebl recording densitometer.

RNA blot hybridization analysis. RNAs were denatured by treatment for 5 min at 65°C in 50% formamide–2.2 M formaldehyde–20 mM morpholinepropanesulfonic acid (MOPS), pH 7.0–5 mM sodium acetate–1 mM EDTA and electrophoresed on 1.5% agarose gels containing 2.2 M formaldehyde–20 mM MOPS, pH 7.0–5 mM sodium acetate–1 mM EDTA. RNA was transferred to nitrocellulose by the procedure of Thomas (44) for 9 h in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) without further treatment of the gel. The nitrocellulose was then air dried and baked for 5 h in vacuo at 80°C. Before hybridization, the nitrocellulose was incubated in a solution consisting of 50% formamide, 5× SSC, 5× Denhardt (0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone), and 200 μg of sonicated salmon sperm DNA (Sigma Chemical Co.) for 4 h at 42°C. Hybridizations were performed at 42°C for 12 to 14 h in the same solution except for a reduction in sonicated salmon sperm DNA to 100 μg/ml and the addition of 20 mM $NaPO_4$, pH 6.5, 10% dextran sulfate (Pharmacia), and 10^6 cpm of labeled nucleic acid per ml. After hybridization, filters were washed three times at room temperature for 10 min in 2× SSC–0.1% sodium dodecyl sulfate and twice at 50°C for 15 min in 0.1× SSC–0.1% sodium dodecyl sulfate. Autoradiographic exposures were made with Kodak XAR-5 film, using Dupont Cronex Lightning-Plus intensifier screens. Autoradiograms were optically quantitated with a Joyce-Loebl recording densitometer. Plasmid DNA was labeled with [^{32}P]dCTP (Amersham) by the nick translation method of Rigby et al. (37).

RESULTS

Growth yield-dependent derepression of APase. P_i is an essential element for yeast growth and fermentation. P_i is assimilated very rapidly, and against an extreme concentration gradient (39), such that APase is derepressed only when exogenous P_i is low and only when growing cells have been subjected to this nutritional limitation. These findings suggest that a tight coupling exists between the utilization of exogenous P_i, the mobilization of endogenous reserves, and the derepression of APase. Our first experiments were designed to explore these relationships. We quantitated the growth yield for strain P28-24C with various amounts of phosphorus and correlated this with the levels of APase derepression (Fig. 1). Biomass was measured by determining the OD_{660} of the culture. As shown in Fig. 1A, in modified Burkholder minimal medium, the growth yield for P_i obeyed a saturation behavior. Above 2 to 3 mM P_i, growth yield was independent of P_i concentration. Below a critical P_i-to-mass ratio, APase was derepressed. This occurred at a P_i concentration of 0.3 mM. Similar data were obtained when the readily metabolizable substrate, β-glycerol phosphate, was used as a source of P_i (data not shown).

To avoid the repression exhibited by growth on P_i or β-glycerol phosphate, a minimal medium containing a more complex organic phosphate supplement was used. This supplement consisted of yeast extract and Bacto-Peptone from which all P_i was removed. The amount of metabolizable organic phosphate in the YEP supplement was determined by measuring cell growth in minimal medium supplemented with various amounts of YEP. Using the growth yield data of Fig. 1A, we calculated this as 0.87 μmol of metabolizable organic phosphate per ml of supplement (data not shown). The levels of enzyme activity at culture densities (OD_{660}) of 0.5 and 1.0 are shown in Fig. 1B. Derepression of APase occurred at higher utilizable phosphate concentrations than was found for P_i (Fig. 1A), indicating a greater growth dependence on APase for generation of an exocellular source of phosphorous from a less available supply.

Physiological transitions during phosphorus starvation. In subsequent experiments, culture conditions were designed such that cell growth would either be independent of APase or dependent upon APase for a supply of phosphorus. The following parameters apply. During growth in high-P_i minimal medium, the P_i requirement is saturated well into the stationary phase, and APase is not synthesized. External P_i is absent in low-P_i minimal medium and is available only from endogenous reserves so that there is essentially no cell growth or enzyme synthesis. In

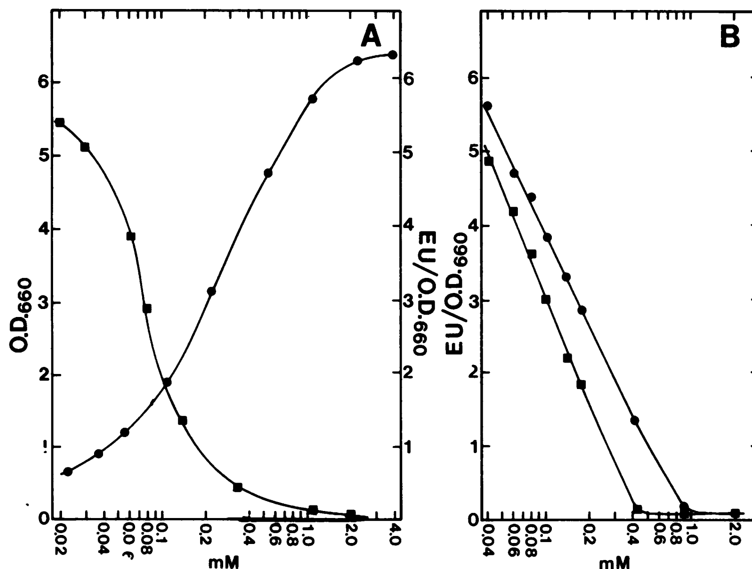

FIG. 1. Effect of phosphate concentration on growth and APase synthesis. Cells of strain P28-24C were grown at 30°C in Burkholder minimal medium at various concentrations of P_i or a low-P_i YEP supplement. (A) Growth in minimal medium; maximum growth (●) or APase activity (EU) at an OD_{660} of 0.5 (■). (B) Growth in minimal medium containing low-P_i YEP (0.872 μmol of utilizable organic phosphate per ml). APase activity was measured at OD_{660} values of 0.5 (■) and 1.0 (●).

low-P_i SMD, P_i is absent, but metabolizable organic phosphate in the P_i-free YEP supplement is supplied at a concentration of 0.17 μmol/ml. Phosphorus is thus available either by consumption of internal reserves, through facilitated transport of the exogenous organic phosphates, or through their degradation by APase, which increases in this medium during P_i-limited growth (Fig. 1B). Upon continuous growth in this medium, the external phosphorus supply is exhausted, accompanied by a period of phosphate-limited growth, after which cells arrest in the G0 state due to P_i starvation. We examined the physiological changes occurring during this transition by measuring the bud index, an indicator of G1 arrest, mean cell volume, cellular RNA, and protein content. Strain Y185 was employed because of an aggregation problem with P28-24C. The onset of APase derepression coincided with the time at which the mean cell volume began to decrease, which occurred at an OD_{660} of 0.21 (Fig. 2). However, departure from exponential growth was observed only above an OD_{660} of 0.9. This decrease in growth rate corresponded with a dramatic increase in the percentage of unbudded cells in the culture. Thus, derepression of APase precedes the onset of G1 arrest by approximately two generations of growth. At the latter time, RNA content per cell markedly decreased, and there was a 90-min delay in further accumulation of APase. These events occurred about one generation before the complete cessation of growth due to P_i starvation. When similar analyses of Y185 were made in the same medium at 23, 26.5, and 34°C, the timing of APase depression varied with the growth rate (data not shown). In all cultures, however, the above parameters held the same relationships, suggesting that it is the yield of growth versus P_i supply, and not rate, that determines the point of derepression.

During the course of these experiments, we noticed that a small but constant amount of APase enzyme activity was found in the culture medium. To our knowledge, the existence of APase external to the cell wall has not been previously reported. To determine whether the presence of enzyme in the medium was a consequence of incubation conditions leading to cell breakage or leakage, we conducted the following experiment. Strain P28-24C was grown in low-P_i SMD medium derepressed for APase, and to one portion of the culture we added P_i to repress futher enzyme synthesis (Fig. 3). To another portion of the culture we added cycloheximide to inhibit further protein synthesis. Both subcultures were incubated with vigorous aeration at 30°C, and soluble and cellular enzyme activities were monitored with time. Upon addition of P_i, total APase activity in the culture increased about 30% over a 30-min period and then remained constant. The increase in enzyme activity occurred with kinetics consistent with a rapid inhibition of transcription and a half-life for

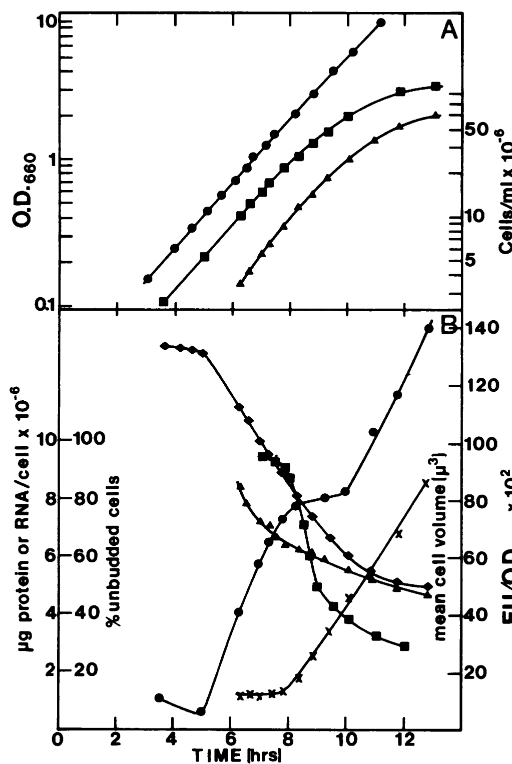

FIG. 2. Effect of phosphate depletion on cellular properties of strain Y185. Cells of strain Y185 were grown overnight at 30°C in high-P_i SMD medium and inoculated at a cell density of 5×10^5 cells per ml into low- or hi-P_i SMD medium. (A) Growth was followed by measuring OD_{660} for high-P_i SMD culture (●) and low-P_i SMD culture (■), or by measuring the cells per ml for the low-P_i SMD culture with a Coulter counter (▲). (B) Changes in cell parameters were measured during growth of the cells in low-P_i SMD medium. Symbols: ●, APase activity (enzyme units [EU]/ OD_{660}); ◆, mean cell volume; ■, RNA per cell; ▲, protein per cell; and ×, percent unbudded cells. See the text for details.

decay of mRNA and for protein maturation of about 8 min (data not shown). The amount of APase in the culture medium rose slowly during growth and increased from 12.3% of the total activity to 36.8% after 6 h. The addition of cycloheximide, however, arrested further accumulation of both the total and soluble APase levels. These data indicate that leakage of the enzyme or cell breakage does not adequately explain the existence of APase in the culture medium, which appears to be a delayed function of de novo enzyme synthesis.

The polypeptide composition of the soluble APase was characterized by labeling derepressed cells with [^{35}S]methionine, purifying the extracellular APase by immunoprecipitation, and treating with endoglycosidase H to remove carbohydrate. The cellular enzyme was similarly treated, and the electrophoretic mobilities of the polypeptides derived from both were compared to each other and to APase polypeptides synthesized in vitro, as described below. As can be seen in the insert of Fig. 3, the soluble APase was indistinguishable from the cellular enzyme. Furthermore, the relative sizes of the in vivo- versus the in vitro-synthesized proteins agreed well with previously published data (8).

Measurement of APase gene transcripts during derepression. The direct measurement of APase gene transcription rates during derepression, by in vivo pulse-labeling methods, was precluded in this work because of the alterations in cell physiology that accompany phosphate starvation, since intracellular pools of P_i, nucleotides, and RNA change significantly. To follow the derepression of the APase genes, we therefore measured instead the total cellular transcript concentrations. Estimation of mRNA half-life was made by measuring mRNA decay after transcriptional repression with P_i. Functional mRNA levels were also determined by using an in vitro protein-synthesizing system. To make these measurements, total cellular RNAs were prepared by phenol extraction and assayed for APase gene transcripts by standard RNA blot hybridization analysis, or for mRNA activity by translation in a wheat germ translation system. Previously, three distinct APase mRNAs (p60, p58, and p56) were identified, which direct the synthesis of four in vivo proteins (D1 through D4) (8). These are shown in Fig. 3 and 4A. Two genes encoding p60 and p56 mRNAs were identified among a collection of phosphate-regulated yeast genes in a λ bank. A yeast DNA fragment containing the p60 gene (24) subcloned into pBR322 (pAP20) was used as a hybridization probe in the RNA blot hybridization experiments reported here (Fig. 4B). The linearity of the hybridization signal and the translational activity with respect to APase mRNA concentration, under our standard conditions, is also shown in Fig. 4. From the translation data, the yield of in vitro-synthesized protein for the three APase mRNAs, as determined by autoradiographic densitometry, was proportional to their specific concentration over a 10-fold range. The same proportionality was observed for hybridization signal and p60 content in RNA blot hybridizations. However, there are two limitations to the use of pAP20 as a probe in this assay. First, in addition to the p60 gene, pAP20 contains a gene for the constitutive APase, p57 (38). Because the p57 gene is not expressed under low-P_i conditions, and only at a low level under high-P_i conditions (Fig. 4B), it contributed negligibly to the hybridization signal. More significantly, the p60 gene has weak complementar-

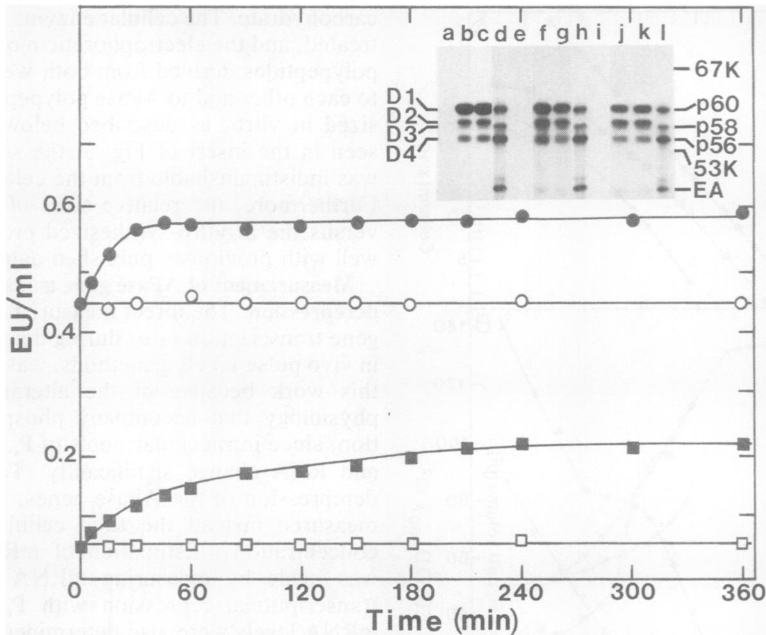

FIG. 3. Effect of cycloheximide and phosphate on APase release. Cells of strain P28-24C were grown at 30°C in low-P_i SMD medium to an OD_{660} of 0.45. The culture was divided, and one portion received 100 μg of cycloheximide per ml (open symbols) and the other 10 μmol of P_i per ml (closed symbols). Cultures were aerated at 30°C, and samples were removed at intervals and assayed for total enzyme activity (EU) (●, ○), or samples were removed, the cells pelleted by centrifugation for 5 min at 8,000 × *g*, and the supernatant assayed for soluble enzyme activity (■, □). Shown in the inset is a portion of an autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel of native and deglycolated intracellular and extracellular APase and the APase polypeptide synthesized in a wheat germ cell-free translation system. Proteins were prepared as described in the text, immunoprecipitated with APase antibody, and either treated or not treated with endoglycosidase H before electrophoresis. Lanes a through c, homogeneous APase preparation; lanes e through g, intracellular APase; and lanes i through k, extracellular APase. Within each set of three lanes, the enzyme preparations were treated as follows: untreated; digested for 2.5 h with 3 μg of endoglycosidase H per ml; or digested for 2.5 h with 3 μg of endoglycosidase H per ml followed by a second addition of enzyme and incubation for 5 h, respectively. Lanes d, h, and l are the in vitro translation products of low-P_i RNA from P28-24C immunoprecipitated with a mixture of APase and enolase immunoglobulin G.

ity to the p58 and p56 mRNAs. By comparing the hybridization signal from P28-24C RNA to that derived from P142-4A, which fails to synthesize p60 mRNA, we estimated that at least 85 to 90% of the hybridization signal is due to reaction with p60 mRNA (Fig. 4B). Since the three APase mRNAs appear to be coordinately regulated (see below), it is unlikely that this percentage changes substantially upon derepression, and certainly not by more than 10 to 15%.

Using the above experimental approach, we measured RNA transcripts and mRNA activity during derepression. Cells were initially grown under repressed conditions in high-P_i SMD medium to mid-logarithmic phase and then transferred to low-P_i SMD medium. In a control culture, cells were transferred into high-P_i SMD medium in the same fashion. Initial growth rates for both cultures were approximately the same for one generation, after which the low-P_i-grown cells displayed a retardation in growth (Fig. 5A). No increase in the basal level of enzyme activity was observed for the control (high P_i) culture (Fig. 5B). However, cells growing in the absence of exogenous P_i showed an elevation in enzyme levels within the first generation of growth before any noticeable alteration in growth rate, within the first 20 min, in fact. After this initial burst of enzyme accumulation (20 to 40 min) was a period of reduced accumulation (40 to 60 min), which preceded a steady-state rate of accumulation of enzyme for most of the duration of the experiment. Because the enzyme is stable under these experimental conditions, for the duration of the experiment (7), the rate of accumulation represents the rate of enzyme synthesis. This was reflected by the dramatic changes in mRNA levels within the first hour. Both p60 transcript and p60 mRNA activity increased rapidly to maxima at 30 min followed by a reduction in

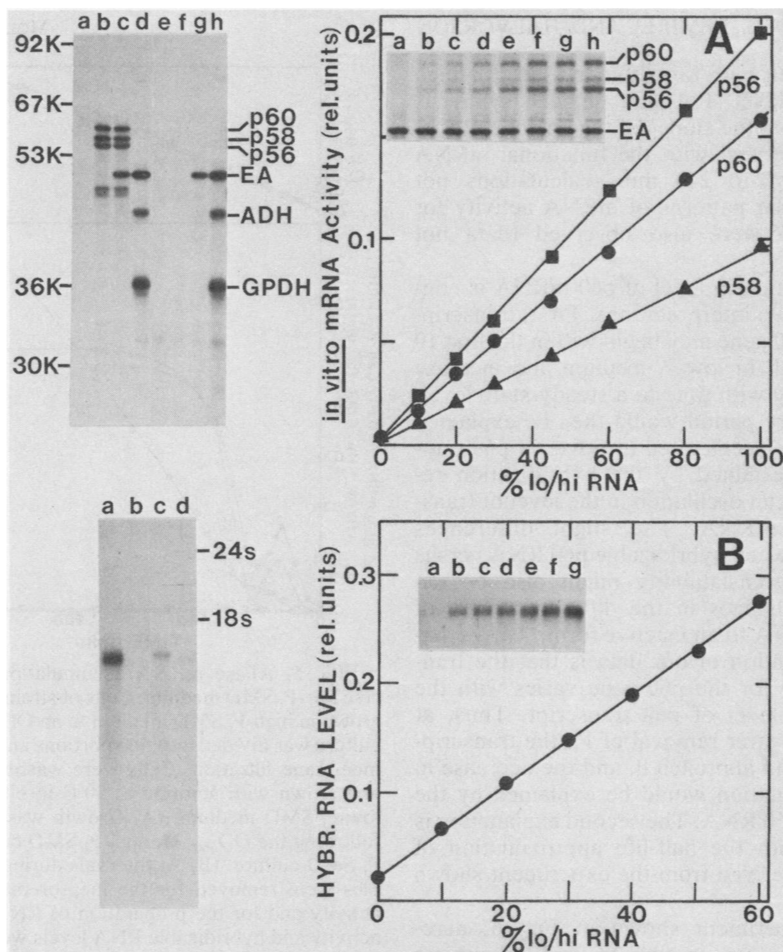

FIG. 4. Measurement of APase gene transcripts and characterization of their in vitro synthetic activity. RNA was isolated from cells of strain P28-24C grown in high- or low-P_i SMD medium to an OD_{660} of 1.0. These total cellular RNA preparations were analyzed (A) by immunoprecipitation of their cell-free translation products with APase immunoglobulin G and other types of control immunoglobulin G or (B) by RNA blot hybridization to a p60 gene probe. (A) Translational analysis. RNAs were translated in a wheat germ cell-free protein synthesizing system with [^{35}S]methionine as described in the text, and the immunoprecipitated translation products were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by fluorography. Shown at the left are the translation products of RNA isolated from cells grown in low P_i (lanes a through d) and high P_i (lanes e through h). The products in each set of lanes were immunoprecipitated with, respectively, preimmune immunoglobulin G; APase immunoglobulin G; a mixture of APase and enolase immunoglobulin G; a mixture of enolase (EA), alcohol dehydrogenase (ADH), and glycerol-3-phosphate dehydrogenase (GPDH) immunoglobulin G. Shown in the inset is a portion of an autoradiogram containing the immunoprecipitated translation products of RNA from low-P_i- and high-P_i-grown P28-24C cells mixed in various proportions: lane a, 0; b, 10%; c, 20%; d, 30%; e, 45%; f, 60%; g, 80%, and h, 100%, low- to high-P_i RNA. The autoradiogram was scanned and quantitated by densitometry, using a Joyce-Loebl recording densitometer. Data were calculated by comparing the yield of specific APase translation products to that of a control RNA used in all subsequent experiments. The yield of polypeptide for a specific APase mRNA was normalized to the corresponding yield of enolase protein for each translation. The enolase mRNA activity remained a constant proportion of the total cellular RNA during changing cellular phosphate concentration in a control experiment. In vitro mRNA activities are expressed as a ratio to the maximal level of APase mRNA activity (total) for RNA isolated from a fully derepressed culture of P28-24C grown in low-P_i SMD medium. (B) RNA blot hybridization analysis. Total yeast RNAs were electrophoresed on a 1.5% agarose–formaldehyde gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pAP20 DNA as described in the text. Shown in the autoradiogram to the left are: lane a and b, P28-24C RNA; and lane c and d. P142-4A RNA; isolated from cells grown in low-P_i (a and c) or high-P_i (b and d) SMD medium, respectively. Shown in the inset is a portion of an autoradiogram of a similar RNA blot of RNA from low-P_i- and high-P_i-grown cells of P28-24C mixed in various proportions: lane a, 0; b, 10%; c, 20%; d, 30%; e, 40%; f, 50%; and g, 60%; low- to high-P_i RNA. The autoradiogram was scanned as in (A), and the amount of RNA hybridized to the p60 gene was expressed in relative density units, as a ratio to the amount of hybridizable RNA in RNA isolated from a fully derepressed culture of P28-24C grown in low-P_i SMD medium.

level, and then both rapidly increased to a high steady-state level. Rates of enzyme synthesis calculated from the slopes of the enzyme plot of Fig. 5B agree well with the functional mRNA data, at least to 240 min (calculations not shown). Similar patterns of mRNA activity for p58 and p56 were also observed (data not shown).

The oscillation in level of p60 mRNA is consistent with two interpretations. First, transcription of the p60 gene may begin within the first 10 min of growth in low-P_i medium and increase hyperbolically with time to a steady-state level. The oscillatory period would then be explained by a period of accelerated turnover of p60 transcript as determined by the hybridization results, causing an oscillation in the level of translatable p60 mRNA. The slight differences observed between hybridizable p60 RNA versus p60 mRNA translatability might also be explained by changes in the differential rate of decay of mRNA to an inactive form. An alternative interpretation of our data is that the transcription rate for the p60 gene varies with the accumulated level of p60 transcript. Thus, at about 30 min after removal of P_i, the transcription rate would approach 0, and the decrease in RNA accumulation would be explained by the turnover of p60 RNA. The second explanation is consistent with the half-life approximation of p60 mRNA derived from the experiment shown in Fig. 6.

In the experiment shown in Fig. 6, derepressed cells were grown to mid-logarithmic phase, and a portion of the culture was repressed by the addition of P_i. At intervals, samples of cells were removed, RNA was prepared by phenol extraction, and mRNA activities were measured as is described in the legend to Fig. 4. Also measured was the level of p60 transcript. The addition of P_i increased the growth rate of the P_i-starved culture (Fig. 6A). While the enzyme level continued to increase in the derepressed culture (Fig. 6B), no enzyme accumulated in the repressed cells 10 to 20 min after addition of P_i. Since the uptake of phosphate from the medium occurs in seconds (39), the subsequent concentration of APase mRNAs is directly related to the rate of repression of transcription and to the rate of decay of mRNA. In all cases, the decreases in concentration were immediate and exponential (Fig. 6C). The decay rate for p60 transcript was 12.6 min, and for translatable p60 mRNA, 8 min. Decay rates for translatable p58 and p56 mRNA were shorter (4.5 and 5.1 min, respectively). The fact that these decay rates were not biphasic, extrapolating to 100% RNA levels at zero time, suggests that the two processes of repression and turnover occur at significantly different rates. From

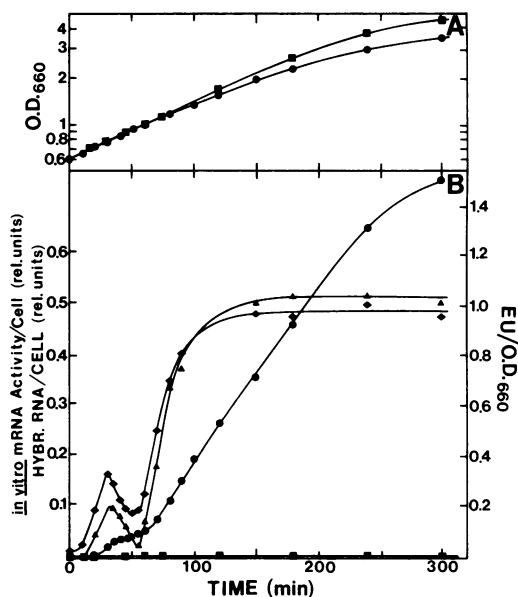

FIG. 5. APase mRNA accumulation in derepressive low-P_i SMD medium. Cells of strain P28-24C were grown in high-P_i SMD medium to an OD_{660} of 0.6. The culture was divided into two portions and harvested by membrane filtration. Cells were washed, suspended, and grown with aeration at 30°C in either high-P_i or low-P_i SMD medium. (A) Growth was monitored by following the OD_{660}: ■, high-P_i SMD culture; ●, low-P_i SMD culture. (B) At intervals during growth, samples were removed for the measurement of enzyme activity and for the preparation of RNA. p60 mRNA activity and hybridizable RNA levels were determined by the procedures used for the experiments described in the legend to Fig. 4. The low-P_i SMD culture was used for measurement of enzyme activity (enzyme units [EU]/OD_{660}) (●), p60 transcript levels (RNA level per cell) (◆), and p60 mRNA levels (in vitro mRNA activity per cell) (▲). The high-P_i SMD culture was used for a measurement of enzyme activity (■). The maximal amount of p60 mRNA activity or hybridizable RNA in cells taken from the low-P_i SMD culture was about 50% of that found for cells fully derepressed in low-P_i SMD medium, as in Fig. 4.

our knowledge of these two processes in other systems, repression is probably instantaneous upon addition of P_i to the culture medium, and mRNA half-lives are probably the actual rates of decay observed here. In any case, these decay rates represent maximum values for the APase mRNAs.

Autoregulation of gene expression involving feedback repression. In the derepression experiment of Fig. 5, there are two potential sources of corepressor for repression of the APase genes, one from endogenous polyP reserves and the other from hydrolysis of the organic phosphate present in the medium. Both methods of gener-

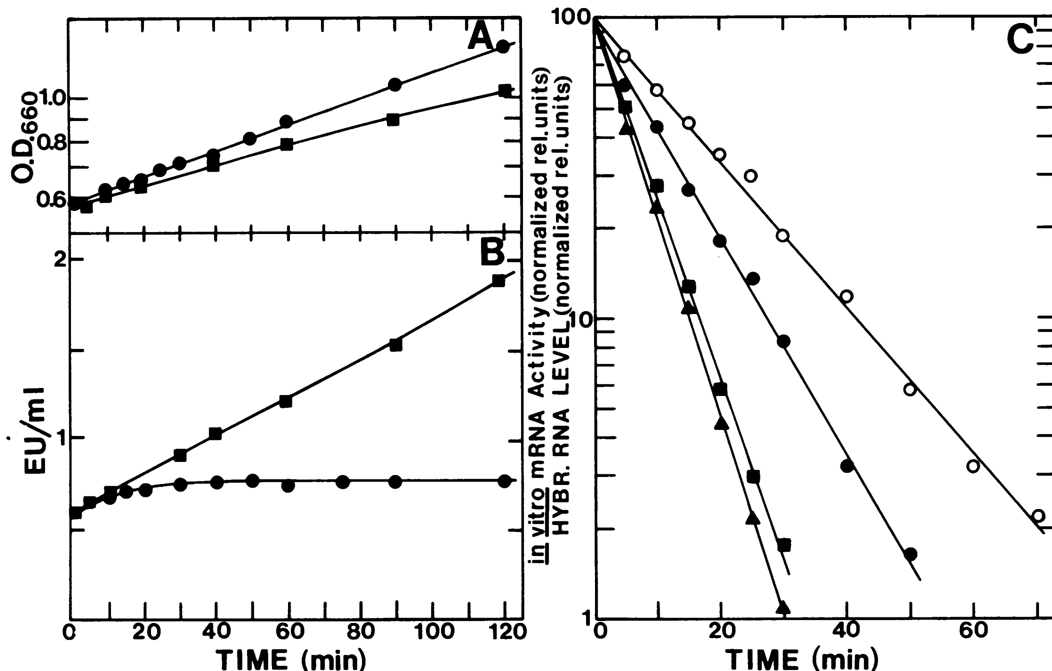

FIG. 6. Estimation of APase mRNA half-lives. Cells of strain P28-24C were grown in low-P_i SMD medium to an OD_{660} of 0.55. The culture was divided, and growth continued either with the addition of 0.81 mg of KCl per ml (derepressed cells) or 1.5 mg of KH_2PO_4 per ml (repressed cells). (A) Growth was monitored by following the OD_{660}: ●, repressed cells; ■, derepressed cells. (B) At intervals during growth, samples were removed and assayed for enzyme activity (EU): ●, repressed cells; ■, derepressed cells. (C) Samples (10 ml) were also removed from the repressed cell culture during growth, and total cellular RNAs were purified as described in the text for the measurement of APase mRNA levels in a wheat germ cell-free translation system: ●, p60 mRNA; ▲, p58 mRNA; ■, p56 mRNA; or, for the measurement of the p60 transcript (○). Data are presented as a percentage of the initial mRNA activity or transcript concentration.

ating corepressor could account for the observed autoregulation of mRNA and transcript levels. It is possible, however, that the oscillation in APase mRNA was brought about by alterations in cell physiology due to filtering the cells and suspending them in fresh medium. To test this possibility, the following control experiment was performed. Cells were grown in high-P_i SMD medium and transferred as before into low-P_i SMD medium or into low-P_i spent SMD medium. To a portion of the latter culture an addition of 1/10 YEP was added after 30 min of growth. Culture OD_{660} values and APase levels were followed as shown in Fig. 7. No oscillation in APase levels was observed for cells suspended and grown in spent medium, whereas addition of YEP 30 min after suspension resulted in an oscillatory pattern very similar to that observed for suspension in fresh medium. This suggests that available P_i from the organic phosphate supplement represses further enzyme synthesis and that enzyme and RNA levels are not perturbed due to experimental design.

Since the regulatory gene products controlling the expression of the p60 gene are expressed constitutively at low and high P_i (51), the generation of a corepressor could have a direct effect on the rate of transcription of the p60 gene. To determine whether hydrolysis of exogenous phosphoester substrate plays a role in the repression of the APase genes through a feedback mechanism, we performed a derepression experiment in which the external source of P_i was eliminated. Repressed cells were transferred into either low-P_i SMD medium or into low-P_i minimal medium (lacking organic phosphate supplement), and enzyme and p60 transcript levels were measured. Cells grew much more slowly in the minimal medium, and, consequently, derepression occurred at a later time. When the data are expressed as a function of growth yield, to normalize differences in growth rates (Fig. 8), we observed that enzyme levels and p60 transcript levels accumulated at lower cell densities in the low-P_i defined minimal medium than in the low-P_i SMD culture. Moreover, the transient decrease in transcript level was reduced. Thus, the majority of the transient reduction in

transcript level in the low-P_i SMD culture was probably due to feedback repression of transcription by the generation of corepressor from the early hydrolytic activity of APase. The observed biphasic accumulation of p60 transcript in the low-P_i minimal medium culture might also be due to a transient repression of transcription through the generation of corepressor from endogenous reserves.

Measurement of endogenous phosphorus pools. The cellular levels of P_i and acid-soluble (mostly PP_i) and acid-insoluble polyP were measured during the course of a derepression experiment as described in the legend to Fig. 5. Cells grown in high-P_i SMD medium were transferred to low-P_i SMD medium and followed during the biphasic period of derepression of APase. Upon derepression, high-molecular-weight PolyP levels rapidly declined just before the iniitial derepression of APase and a surge in acid-soluble polyP (Fig. 9). Both the acid-soluble and -insoluble polyP fluxed during the lag in APase accumulation before exhaustive derepression. Most interesting were the changes in P_i levels. The initial rise coincided with the decrease in polyP reserves, and the second rise coincided with the first derepression of APase. Throughout the remainder of the experiment P_i levels remained high, even during the second phase of APase derepression, and thus they may not serve as corepressor for the phosphatase genes. The inverse correlation of APase levels with acid-soluble polyP (primarily PP_i) suggests that PP_i or a low-molecular-weight polyP may serve as a regulatory function for APase.

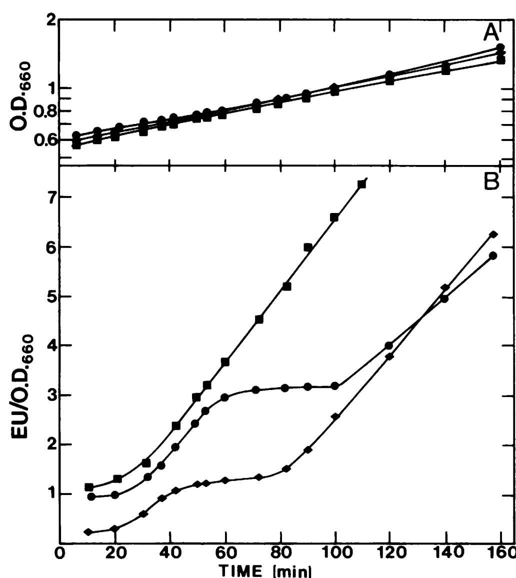

FIG. 7. APase derepression in spent low-P_i SMD medium. Repressed cells of P28-24C were grown in high-P_i SMD medium as described in the legend to Fig. 5, harvested by filtration, and suspended in either fresh or spent low-P_i SMD medium to an initial OD_{660} of 0.5. Spent medium was obtained by filtering a culture of low-P_i SMD-grown cells of P28-24C at an OD_{660} of 0.5. (A) Growth of cultures monitored by OD_{660}. (B) Enzyme activity (enzyme units [EU]/ OD_{660}): ◆, fresh low-P_i SMD culture; ■, spent low-P_i SMD culture; ●, spent low-P_i SMD culture to which 1/10 YEP was supplemented after 30 min of growth.

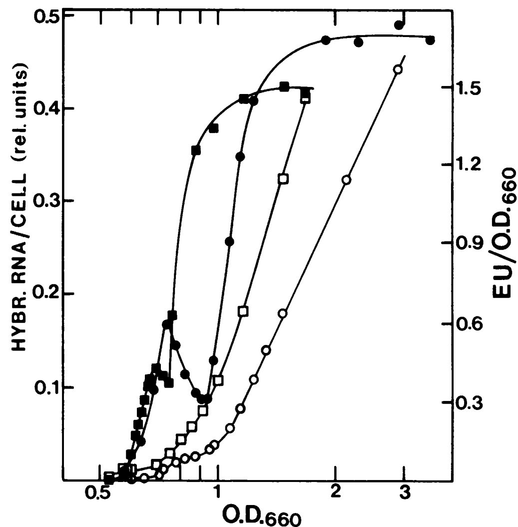

FIG. 8. Comparison of enzyme and p60 transcript levels during derepression in minimal and SMD medium. Cells of strain P28-24C were grown in high-P_i SMD medium to mid-logarithmic phase and transferred to low-P_i SMD medium and low-P_i minimal medium as described in the legend to Fig. 5. At intervals during growth, samples were removed for measurement of enzyme activity (enzyme units [EU]/ OD_{660}): ○, low-P_i SMD medium culture; □, low-P_i minimal medium culture. For measurement of p60 transcript levels (relative hybridizable RNA level per cell) by the procedures described in the legend to Fig. 4 (see the text): ■, low-P_i minimal medium culture; ●, low-P_i SMD medium culture. Data are presented as a function of culture OD_{660}.

Physiologically integrated transcriptional controls. To determine whether the external source of corepressor for the APase genes is integrated physiologically with the cyclic pool of P_i and polyP (as suggested by the data of Fig. 9), we examined the influence of endogenous reserves on the accumulation kinetics of the p60 transcript and mRNA activity during derepression by pregrowing cells under repressed conditions at different P_i concentrations (preculture P_i) before the onset of derepression. Lowering the preculture P_i concentration lowers the size of the polyP pool (18). Cells were pregrown as

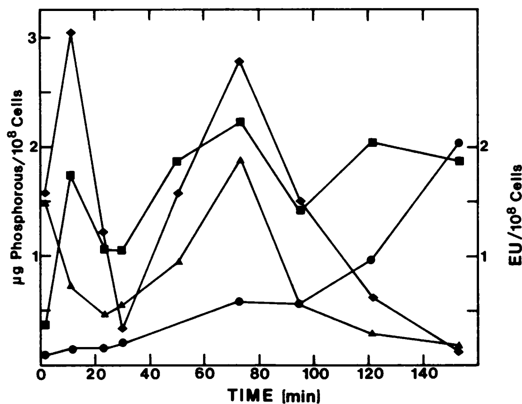

FIG. 9. Measurement of intracellular polyP and P_i pools during derepression. Cells of strain P28-24C were grown in high-P_i SMD medium to an OD_{660} of 0.5. Cells were harvested by membrane filtration and transferred to low-P_i SMD medium. At intervals during growth, samples were removed for the measurement of APase activity (enzyme units [EU] per cell) (●), P_i (■), acid-soluble polyP (◆), and acid-insoluble polyP (▲).

before in high-P_i SMD medium (2.2 mM P_i), in SMD medium containing 0.22 mM P_i, which just saturates the P_i requirement for repression throughout the experiment, and in SMD medium containing an intermediate concentration of P_i (0.55 mM). Derepression of the cells and measurement of p60 transcript and mRNA activity were accomplished as described above. Growth rates were about the same for all three derepressed cultures (Fig. 10A). Enzyme activity appeared in all three cultures at about the same time, but enzyme levels were higher at increasingly higher concentrations of P_i in the pregrowth medium (Fig. 10B). Lowering the pregrowth P_i concentration caused a reduction in the amount of early p60 transcript accumulation and slightly delayed the time of maximal increase in p60 transcript levels (Fig. 11A). A similar response was observed for all three mRNA activities (Fig. 11B through D). Since the amount of external organic phosphate was the same for all three cultures and since the growth rates for all three cultures suggest that the organic phosphate is being utilized, then the differences in levels of mRNA must be due to differences in the utilization or sequestration of intracellular P_i. Regardless of the actual mechanisms that autoregulate p60 mRNA levels, expression of the p60 gene is clearly influenced by, and perhaps determined by, the metabolic balance of the polyP cycle. The precise nature of the controls governing the utilization of the endogenous and exogenous reserves, however, remain to be determined.

DISCUSSION

In this paper we have examined the regulatory controls affecting APase expression at the physiological level by a series of repression and derepression experiments. We designed culture conditions of phosphate-limited growth and metabolism that led to APase depression, and then followed the regulation of the APase genes at the molecular level. The results represent one of a few examples in eucaryotic cells where physiological controls have been documented at the level of RNA regulation. We have shown that APase synthesis is tightly regulated throughout the growth phase. During exponential growth in the presence of external P_i, the *PHO5* gene is repressed (Fig. 5). As external P_i becomes limiting, APase is derepressed, leading to a biphasic appearance of p60 mRNA and APase (Fig. 5, 7, 8, 10, and 11). This transient reduction in mRNA levels is explainable by a number of mechanisms, such as inhibition of transcription or

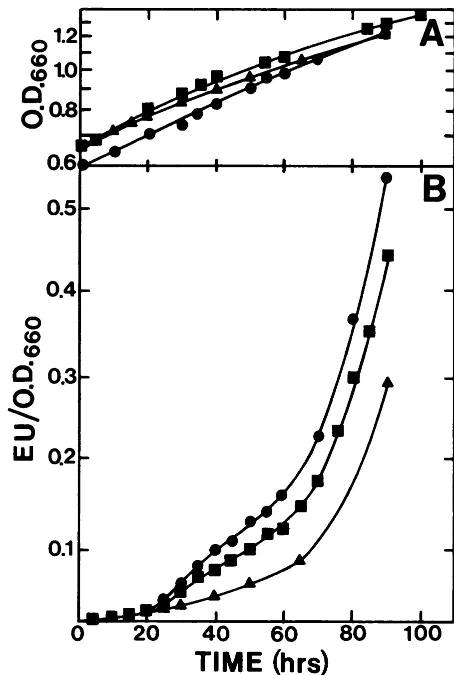

FIG. 10. Effect of various pregrowth concentrations of P_i on APase accumulation during derepression. Cells of strain P28-24C were grown in SMD medium containing: ●, 2.2 mM P_i; ■, 0.55 mM P_i; or ▲, 0.22 mM P_i. When the culture reached an OD_{660} of 0.7, cells were harvested by membrane filtration, washed with low-P_i SMD medium, suspended in the same medium, and grown at 30°C with aeration. (A) Growth was monitored by following the OD_{660} of cultures. (B) At intervals, samples were removed and assayed for enzyme activity, expressed as enzyme units (EU) per OD_{660}.

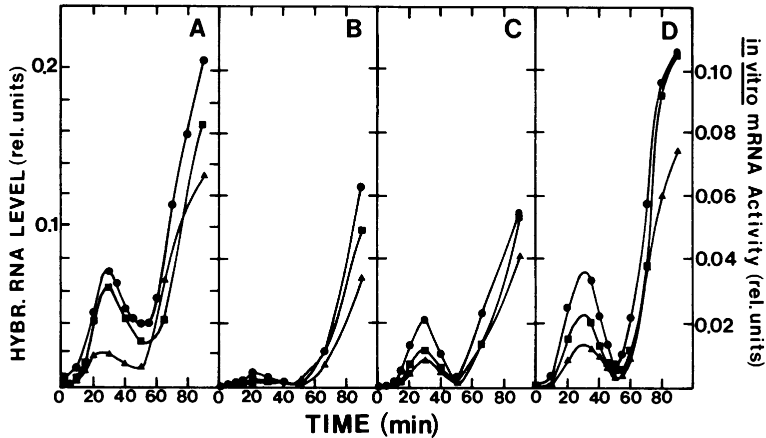

FIG. 11. Effect of pregrowth concentrations of P_i on APase mRNA accumulation during derepression. The cultures from the experiment shown in Fig. 10 were also analyzed for p60 transcript levels and for APase mRNA levels by the procedures described in the legend to Fig. 4 (see the text). (A) p60 transcript level (relative hybridizable RNA concentration) for cultures pregrown in: ●, 2.2 mM Pi; ■, 0.55 mM P_i; and ▲, 0.22 mM P_i. (B), (C), and (D) show the mRNA activity in total cellular RNAs for: ▲, p60 mRNA; ■, p58 mRNA; and ●, p56 mRNA determined in a wheat-germ cell-free translation system for culture pregrown in 0.22 mM P_i (B), 0.55 mM P_i (C), and 2.2 mM P_i (D).

changes in mRNA turnover. The latter mechanism has been observed in several eucaryotic systems (1). Our data on mRNA half-lives are in agreement with a mechanism of transcriptional autoregulation. Such a model is also consistent with the turnover of intracellular polyP that occurs during derepression. The accelerated degradation of polyP upon P_i starvation could provide a limited source of corepressor for the APase genes and thereby lower their transcription rates. Cellular demands on P_i for growth would shortly exhaust this supply of corepressor, and transcription of the APase genes would continue. The effect of preculture P_i concentration on APase derepression (Fig. 10 and 11) suggests that the polyP cycle controls the level of corepressor. When starved cells are completely derepressed, the majority of the transcription of p60 mRNA occurs within 0.1 of a cell generation (Fig. 5 and 8). The maximal linear rate of APase synthesis is observed only after this point when the amount of p60 mRNA per cell is constant. Before this point, APase synthesis is subject to feedback repression of transcription determined by endogenous phosphate reserves (Fig. 8). Such transitory kinetics during autoregulation have been recognized at the enzyme level in eucaryotes for some time (17). The precise nature of the controls governing the utilization of the endogenous and exogenous reserves, however, remains unclear. Since APase regulation is closely correlated with acid polyP levels (primarily PP_i) (Fig. 9), the role of P_i as corepressor is questionable. Observations that PP_i can serve as an energy source for certain microorganisms and that low-molecular-weight polyP can regulate RNA synthesis in fungi and the polysaccharide content of the cell wall in yeasts (for review, see reference 25) give credence to its potential role as a regulatory signal.

Both the function and evolutionary divergency of the closely related APase genes is unknown. As shown in this paper, the three repressible APase genes are coordinately regulated in a similar fashion during both repression and derepression. All three APase polypeptides are processed, glycosylated, and active enzymatically, and there are no observable differences in the regulated expression of the mRNA levels. Measurable differences were observed, however, in the functional mRNA half-lives of p60, p58, and p56 mRNA (Fig. 6C). The half-lives reported here agree well with the reported chemical half-lives of mRNAs for β-galactosidase (26), iso-1-cytochrome *c* (51), and orotidine-5′-phosphate decarboxylase (3) or the synthetic capacity mRNA half-lives of arginase (5), allophane hydrolase (27), or ornithine carbamoyl transferase (34). The changes that occur in physical levels of p60 during derepression agree well with the translational activity of p60 mRNA in the wheat germ cell-free translation system (Fig. 5). Differences are observed between translatability and p60 mRNA levels at the early transient stage of derepression, which may be explained by differences in physical and functional mRNA half-lives. Although these as-

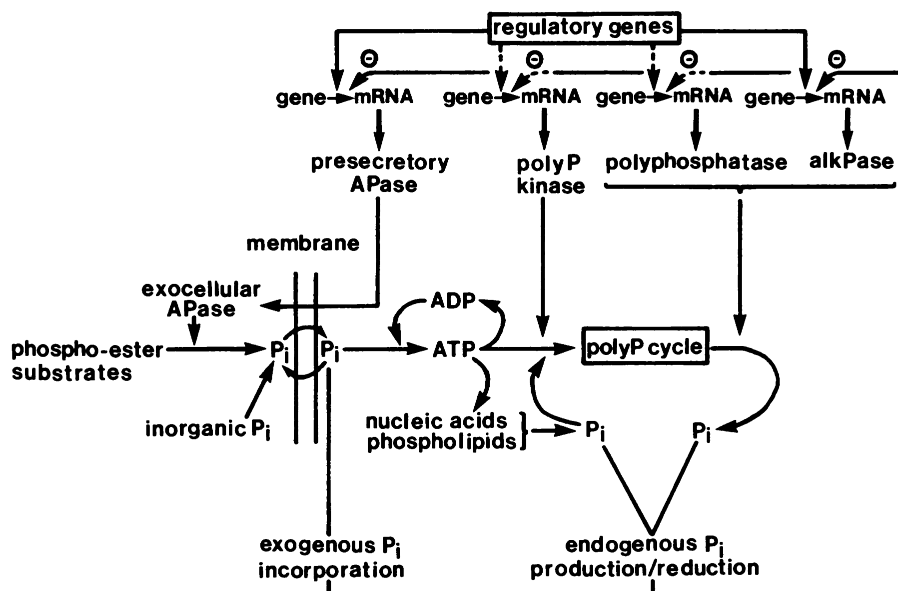

FIG. 12. Model for phosphate metabolism and phosphatase regulation in *S. cerevisiae*.

pects of the derepression process are not yet understood, our data nonetheless indicate that there is not a long delay in mRNA processing.

From the data presented here and elsewhere, we have constructed a scheme for phosphorus metabolism and phosphatase regulation in *S. cerevisiae* (Fig. 12). Accordingly, the cyclic regeneration of ATP from ADP yields energy for metabolism as well as ATP for biosynthetic pathways. ATP, ADP, and P_i concentrations are controlled through the polyP cycle, both for efficient energy utilization at high phosphate transfer potentials for ATP and also for modulation of their function as allosteric effectors in numerous metabolic reactions (12).

The major enzymes of the polyP cycle, as shown, are polyP kinase, polyphosphatase, and alkaline phosphatase. PolyP kinase is involved in the synthesis of polyP but probably not in its degradation (29, 30, 48). The sequential hydrolysis of polyP to the smaller residues, and ultimately to P_i, occurs via polyphosphatase (10), and by alkaline phosphatase, vacuolar enzyme (21). Cells growing under nonlimiting conditions of P_i change polyP levels with changing physiological requirements (22). During exponential growth, nucleic acid and phospholipid synthesis inhibits polyP deposition and stimulates its degradation so that little polyP accumulates. As growth rate declines, degradation is inhibited and polyP accumulates (22). Thus, cycling of polyP has the effect of balancing cellular levels of P_i with the energy charge of the cell, as defined by Atkinson (2), with the regeneration of ATP for biosynthesis.

Phosphate starvation leads to an apparent derepression of all of the enzymes shown in Fig. 12 (10, 18). The derepression of exocellular APase results in the generation of P_i from available external phosphoester substrates (41, 46) followed by its intracellular incorporation. Derepression of the other enzymes results in polyP degradation (16, 49) followed by utilization of the liberated P_i in the biosynthesis of nucleic acids and phospholipids (18). Thus, two potential precursor pools of P_i are available upon derepression. The molecular mechanisms regulating the expression of these enzymes, however, are unknown. The genetic system (45, 46) regulating APase expression at the transcriptional level also participates in the regulation of alkaline phosphatase (21) and perhaps in the expression of other phosphorus-metabolizing enzymes. The autoregulation of APase expression that occurs during derepression may also use this genetic system at the transcriptional level, which should be testable in future studies by analysis of temperature-sensitive regulatory gene mutants.

Changes in phosphate flow and utilization have also been observed in synchronous cultures of *S. cerevisiae* (15, 16). As in asynchronous cultures, synchronously dividing cells consume polyP only in the absence of an adequate supply of exogenous P_i. In the period during initiation of DNA synthesis, external P_i rapidly decreased with a simultaneous increase in polyP. In the following period of DNA synthesis (S period), if the external P_i level is low, polyP is consumed, presumably by acting as a substitute

source of phosphate in place of external P_i (15, 16). During this same period of the cell cycle, APase has been shown to have a step-wise increase in activity (33) associated with the morphological changes occurring during bud emergence (13, 28). We have observed similar periodic increases in APase activity in our own cell cycle studies, concomitant with bud formation, and have correlated these changes with changes in APase mRNA levels (50). The changes in concentrations of polyP and intracellular P_i occuring during this period of the cell cycle may play an important role in the periodic expression of APase. Moreover, a study of these phenomena may reveal mechanisms whereby P_i and polyP regulate the expression of APase.

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