Proc. Natl. Acad. Sci. USA
Vol. 77, No. 8, pp. 4504–4508, August 1980
Biochemistry

In vitro synthesis of repressible yeast acid phosphatase: Identification of multiple mRNAs and products

(glycosylation/eukaryotic gene expression/peptide mapping)

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Communicated by Martin Gibbs, April 28, 1980

ABSTRACT **Antibodies to repressible nonspecific acid phosphatase [APase; orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2] purified from *Saccharomyces cerevisiae* were used to detect the *in vitro* products of APase mRNA. Immunoprecipitation of cell-free synthesized protein and of *in vivo* enzyme from cell extracts has shown that derepression of enzyme synthesis *in situ* is the result of *de novo* appearance of functional mRNA followed by *de novo* protein synthesis. At least three unique APase polypeptides are synthesized *in vitro* from separate mRNAs and appear to be glycosylated *in vivo* to form secreted enzyme.**

Phosphorus metabolism in *Saccharomyces cerevisiae* depends upon several constitutive and phosphate-repressible acid and alkaline phosphatases (1–3). Their expression is governed by a complex but genetically well-defined eukaryotic dispersed-gene control system (4). The repressible yeast acid phosphatase [APase; orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2], an enzyme similar to most bacterial and higher eukaryotic alkaline phosphatases, is an exocellular, facultative glycoprotein that scavenges phosphorus for the growing cell (5). Its synthesis probably involves transcriptional control of the structural gene *PHOE* (6) by a number of regulatory genes mediated by inorganic phosphate, although this model has recently been questioned (7). Major interests in repressible yeast APase originate from its cell cycle-dependent expression (8), its association with bud emergence (9, 10), its highly integrated secretion which is similar to that of glycoproteins in higher mammalian systems (9, 10), and its coordinated differential expression with the nonglycosylated, cytoplasmic, alkaline phosphatase (1, 3, 4).

We have initiated a study of the yeast phosphatase system by characterizing the metabolism of repressible APase mRNA in an *in vitro* assay system. Cell-free synthesized proteins were identified in translations programmed with total yeast mRNA by immunoprecipitation with antibodies to the repressible enzyme. In this paper we describe experiments which characterize three mRNAs coding for three antigenically related APase polypeptides that have different but substantially homologous sequences. By analysis of several structural gene (*phoE*) mutants, we have shown that at least one of these polypeptides is glycosylated protein with enzyme activity. The precise relationship among the three proteins and among the three mRNAs is not clearly defined, but we show that all three mRNAs are found on polysomes, that their activities *in vitro* reflect cellular synthetic capacity, and, furthermore, that derepression of APase results in the *de novo* appearance of these mRNAs and their *in vivo* products.

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MATERIALS AND METHODS

Yeast Strains and Media. The haploid yeast strain H42 (ATCC 26922: a *gal4 PHOC PHOE*) has the wild-type genotype for the production of repressible and constitutive acid phosphatases. P28-24C (*a phoC-1*), lacking the constitutive enzyme, and P142-4A (*α phoC-1 arg phoE*) and 021-MA (*α phoC-1 phoE-2*) lacking both enzymes were derived by mutagenesis of strain H42. These were kindly provided by Akio Toh-e. Haploid strain 108-3C (*a trpl ural*) was from Howard Douglas and A430 (*a met6*) was from James Haber. SMD medium (pH 4.7), consisted of modified Burkholder minimal medium (2) and one-fifth strength low-P_i YEP medium (11), containing 2% (wt/vol) dextrose and 1.5 g of KCl (low P_i) or KH_2PO_4 (high P_i) per liter.

Cell Growth, Isotopic Labeling, and Preparation of Cell Extracts. Cells grown in SMD medium at 30°C to a density of 10^6/ml were steady-state labeled with L-[^{35}S]methionine (>800 Ci/mol; 1 Ci = 3.7×10^{10} becquerels; Amersham/Searle) for 6.5 generations at an initial concentration of 7 μCi/ml. Extracts were made from 10-ml cultures after addition of 50 μg of cycloheximide per ml. Cells washed once with BSB buffer (0.01 M $Na_2B_4O_7$/0.15M NaCl, pH 8.0) were disrupted in 0.3 ml of buffer containing 0.1% $NaDodSO_4$ by mixing for 45 sec with 0.67 vol of 0.45-mm glass beads on a vortex mixer. Extracts were recovered from the beads by three 0.25-ml washes and clarified by centrifugation at $8000 \times g$ for 20 min.

Purification of Repressible APase. Repressible APase was obtained from P28-24C, P142-4A, and A430 grown as described above with 3 μCi of [^{35}S]methionine per ml, and from strain 108-3C without isotopic label. Cells washed once with 0.1 M NaOAc buffer (pH 4.2) were resuspended in 1.5 vol of buffer and disrupted with glass beads (11). The enzyme was then purified to homogeneity by the method of Boer and Steyn-Parve (12) with only minor modification. Wild-type enzyme had a specific activity of 40.5 enzyme units/mg of protein when assayed spectrophotometrically with *p*-nitrophenyl phosphate (2). On electrophoresis, the enzyme moved toward the anode at pH 6.5 or 4.3 as a single sharp glycoprotein band staining for enzyme activity. No protein was detected with reversed electrodes. It also sedimented as a single protein band of constant specific activity when ultracentrifuged on 7.5–25% (wt/vol) sucrose gradients.

Preparation and Size Fractionation of RNA. Total cellular RNAs from midlogarithmic-phase cells ($2–5 \times 10^7$ cells/ml) were obtained after cell disruption by standard H_2O-saturated phenol extraction, and the poly(A)-containing mRNA was purified by oligo(dT)-cellulose chromatography (11). Polysomal mRNA was obtained by phenol extraction of polysomes (11).

Abbreviations: APase, acid phosphatase; HPLC, high-pressure liquid chromatography.

The preparative size fractionation of yeast poly(A)-containing mRNA by continuous elution mercurial-agarose gel electrophoresis and analysis of functional mRNA in the eluted fractions has been described (13).

Cell-Free Protein Synthesis. Total yeast cellular, polysomal, and poly(A)-containing mRNA were routinely translated in wheat embryo or reticulocyte systems as described (11, 13), optimized for concentrations of various components and new lysate. Translation products were analyzed and detected by fluorography after $NaDodSO_4$/polyacrylamide gel electrophoresis by published methods (11, 13).

Immunoprecipitation and Immunocompetition Assays. Antibodies to repressible APase, and to several yeast enzymes as controls, were made in New Zealand White rabbits, and the IgG purified from immune sera was tested by double-diffusion Ouchterlony assay (11, 13). Each formed single precipitin bands against the original pure antigens. Solid-state immunoprecipitations were performed with protein A-Sepharose CL-4B (Pharmacia) in BSB buffer containing 1 mg of bovine serum albumin per ml, 0.05% $NaDodSO_4$, and 0.1 M methionine. Reaction mixtures contained (in 125 μl) 10 μl of translation mixture plus 10 μg of immune rabbit IgG or 20–30 μg of radiolabeled protein (1.5×10^4 cpm/μg) from cell extracts and 20–40 μg of IgG, with total protein adjusted to 1 mg/ml with albumin. After 1 hr at 37°C, 25 μl of a 20% (vol/vol) suspension of swollen protein A-Sepharose in BSB was added. After incubation for 30 min at 4°C on a rotating drum, the reaction mixtures were diluted to 5 ml with BSB containing albumin (0.3 mg/ml), 0.1 M methionine, and 0.01% $NaDodSO_4$, and the beads were collected by centrifugation (2500 rpm, 10 min, 4°C). Bound protein was dissociated by heating at 100°C for 4–5 min in 100 μl of double-length Studier's buffer (11). Specificity of the immunoprecipitation reactions was determined by competition experiments using nonradioactive pure protein. These differed from the above by a 5-min preincubation of the IgG with 0.1–10 μg of the competing antigen immediately prior to addition of the cell extract or translation mixture.

Protein Characterization. Peptide maps were obtained by high-pressure liquid chromatography (HPLC) of tryptic digests of electrophoretically purified proteins as described (11) and by partial proteolysis with *Staphylococcus aureus* V8 protease by the method of Cleveland *et al.* (14). Deglycosylation by endoglycosidase H digestion after iodoacetamide treatment was according to Rothman *et al.* (15) or by the HF procedure of Mort and Lamport (16).

Genetic Analysis. Heterozygous diploids for the *phoE* mutation were obtained and tetrad analysis was performed by standard genetic techniques as described (2).

RESULTS

Inorganic Phosphate Repressed Synthesis of APase. To determine the repressibility of APase synthesis, immunoprecipitations were performed on total soluble protein extracts from [^{35}S]methionine-labeled cells grown in low P_i or high P_i medium. A number of constitutive enzymes (glyceraldehyde-3-phosphate dehydrogenase, enolase, alcohol dehydrogenase, and transketolase) served as internal controls for uniform labeling and recovery of protein. Autoradiography of the electrophoretically fractionated immunoprecipitates revealed that the control radiolabeled polypeptide levels were unchanged by the different cell growth conditions (Fig. 1*A*). However, as seen, the APase glycoprotein was detectable only in derepressed cell extracts (low P_i) and not in extracts from repressed cells (high P_i). The latter growth condition led to almost complete repression of APase activity. IgG from preimmune serum did not react with any of these polypeptides. Furthermore, reactivity of APase IgG with other nonspecific glycoproteins present in the extracts was minimal as judged by: (*i*) the lack of immunocompetition with saturating amounts of nonradioactive

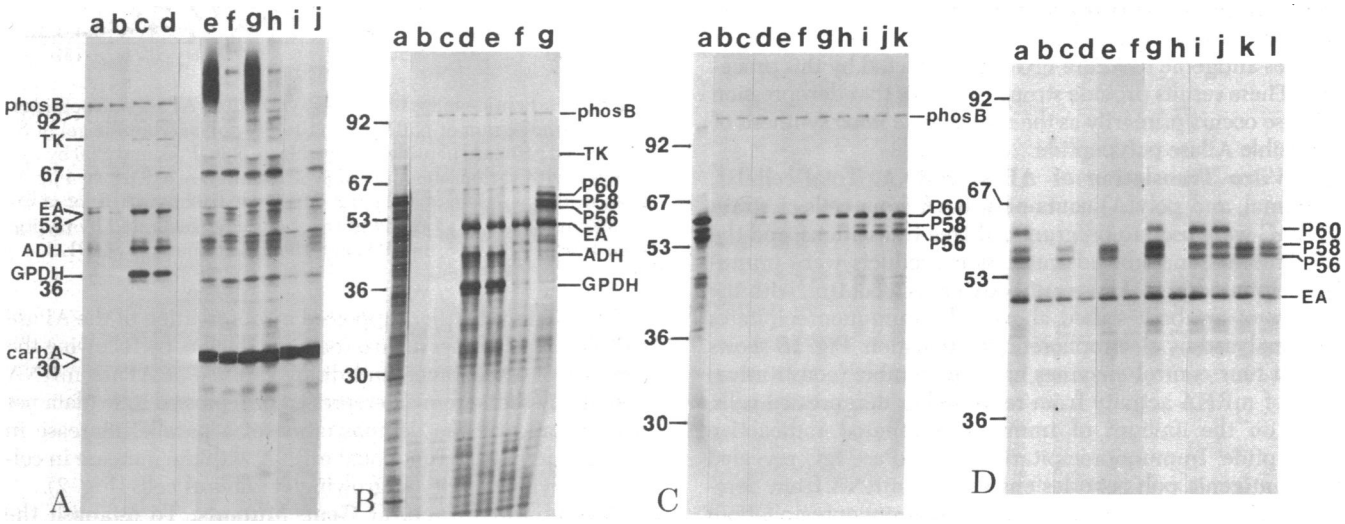

FIG. 1. Composite gel autoradiograms of *in vivo* and *in vitro* derived [^{35}S]methionine-labeled proteins. (*A*) Lanes: a and b, derepressed and repressed cell extracts immunoprecipitated with preimmune IgG; c and d, derepressed and repressed cell extracts immunoprecipitated with a mixture of IgGs to transketolase (TK), enolase (EA), ADH, and GPDH; e and f, derepressed and repressed cell extracts immunoprecipitated with APase IgG; g and h, as in e and f plus 4 μg of nonradioactive invertase added to the immunoprecipitation reaction; i and j, as in e and f plus 1 μg of nonradioactive APase added to the immunoprecipitation reaction. Numbers identify molecular weights of size markers $\times 10^{-3}$. (*B*) Lanes: a, total translation products of RNA from derepressed cells of strain P28-24C; b–g, translation products of mRNA from repressed (lane b) and derepressed (lane c) cells immunoprecipitated with preimmune IgG, from repressed (d) and derepressed (e) cells immunoprecipitated with a mixture of IgGs to transketolase (TK), enolase (EA), ADH, and GPDH, and from repressed (f) and derepressed (g) cells immunoprecipitated with APase IgG. (*C*) Translation products of mRNA from derepressed cells immunoprecipitated with APase IgG (a) or preimmune IgG (b). Lanes c–k are immunoprecipitations identical to lane a except for the addition of 2 μg (c), 1 μg (d), 0.5 μg (e), 0.2 μg (f), 0.1 μg (g), and 0.05 μg (h) of nonradioactive APase or 2 μg (i), 0.5 μg (j), or 0.1 μg (k) of nonradioactive invertase. (*D*) Immunoprecipitated translation products of mRNA from derepressed (a) and repressed (b) cells of P28-24C, derepressed (c) and repressed (d) cells of P142-4A, derepressed (e) and repressed (f) cells of 021-MA, derepressed (g) and repressed (h) cells of a diploid (BL3) derived from the cross of P28-24C and P142-4A, derepressed cells of *PHOE* segregants (i and j), and derepressed cells of *phoE* segregants (k and l) derived from tetrad dissection of the diploid BL3 of lane h.

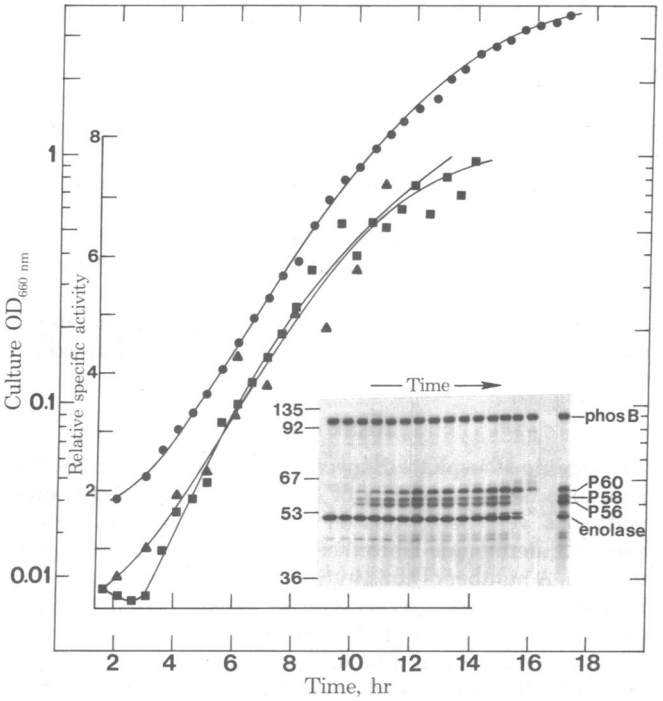

FIG. 2. Correlation of APase mRNA and enzyme activities with growth. Cultures of strain P28-24C grown at 30°C in low-P_i SMD medium were assayed for cell density (●; by OD_{660}), enzyme activity (■; units/unit cells), and *in vitro* mRNA activity (▲; P60 mRNA activity/μg RNA). (*Inset*) Gel autoradiogram showing APase and enolase immunoprecipitated *in vitro* translation products of the RNA isolated at various times, which were used for the densitometric determination of P60 mRNA (▲). [^{14}C]Phosphorylase B was added as a norm.

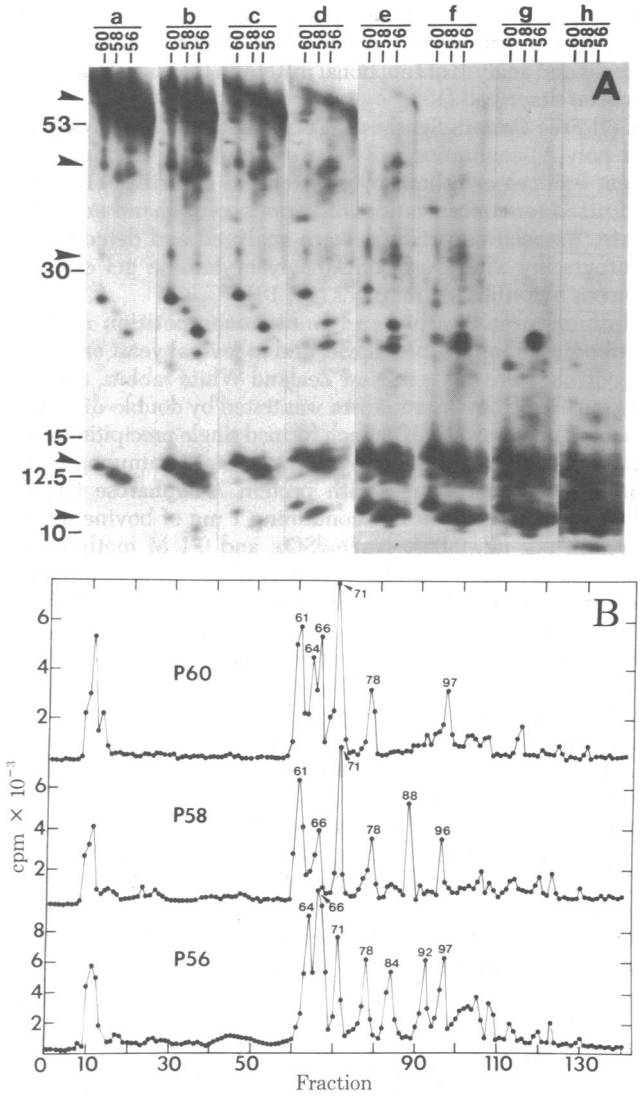

FIG. 3. Peptide maps of *in vitro* synthesized APase polypeptides. (*A*) Autoradiogram of $NaDodSO_4$/10–15% polyacrylamide gel of *S. aureus* V8 protease peptides derived from P60, P58, and P56 by hydrolysis with various amounts of protease. Lanes: a, 8 μg; b, 4 μg; c, 2 μg; d, 1 μg; e, 0.4 μg; f, 0.2 μg; g, 0.04 μg; h, 0.02 μg. (*B*) Plot of radioactivity versus fraction number for peptides generated by tryptic digestion of P60, P58, and P56 and then fractionated by HPLC.

invertase glycoprotein (Fig. 1*A*); (*ii*) the specificity of immunocompetition with nonradioactive APase (Fig. 1*A*); and (*iii*) indirect immunocompetition experiments with the cell-free translation products described below (data not shown). No other peptides antigenic to APase IgG were detected by this procedure. These results provide strong evidence that derepression of APase occurs primarily as the results of *de novo* synthesis of repressible APase polypeptide.

***In Vitro* Translation of APase mRNA.** Total cellular, polysomal, and poly(A)-containing RNA from cells of strain P28-24C were used to program cell-free translations, and the L-[^{35}S]methionine-labeled translation products were immunoprecipitated with APase antibody or, as a control, with the mixture of antibodies used above. The immunoprecipitates were analyzed by electrophoretic fractionation. Fig. 1*B* shows that all four control enzymes had comparable (constitutive) levels of mRNA activity from repressed or derepressed cells, based on the amount of immunoprecipitated radioactive polypeptide. Immunoprecipitation with APase IgG revealed three antigenic polypeptides encoded by mRNA from derepressed cells that were absent in control immunoprecipitations with preimmune IgG. We have designated these as P60, P58, and P56, corresponding to their approximate size in kilodaltons. All three were shown to be specific to APase by radioisotope dilution immunocompetition experiments with nonradioactive antigen and by the lack of competition with invertase glycoprotein (Fig. 1*C*).

None of these polypeptides were synthesized from mRNA from cells grown under phosphate repressed conditions (Fig. 1*B*). Consistent with our *in vivo* observation for the *de novo* synthesis of repressible APase polypeptide (Fig. 1*A*), this strongly indicates that derepression of acid phosphatase is based on the *de novo* appearance of functional repressible APase mRNA. This is further supported by observation of the APase mRNA on polysomes *in vivo* (data not shown), by following the kinetics of appearance and disappearance of APase mRNA activity by shifts from a derepressed or repressed state (data not shown), and by the demonstration of a parallel increase in specific activity of functional mRNA with the increase in cellular enzyme activity in growing cultures of cells (Fig. 2).

Analysis of Structural Gene Mutants. To establish the identity of the *in vitro* synthesized, immunoprecipitated proteins, we analyzed the mRNA activities of several repressible APase structural gene (*phoE*) mutants. In three independently isolated mutant alleles studied, we observed a complete lack of P60 mRNA activity but wild-type levels of P58 and P56 mRNAs (Fig. 1*D*). In one mutant (021-MA) this was accompanied by the appearance of a new immunoprecipitable peptide slightly shorter than P58, suggesting that this allele is a chain termination (nonsense) mutant affecting the product, P60, of the *PHOE* gene. To confirm that this alteration in P60 is the result of the mutation in the *PHOE* gene, and thus to confirm that P60 is a *PHOE* product, we crossed O21-MA with

the wild-type strain P28-24C and subjected the diploid to tetrad analysis. As shown in Fig. 1*D*, a gene dosage effect was observed in relation to the three mRNA activities in the haploid strains vs. the diploid. In all 10 tetrads analyzed, the P60 mRNA minus phenotype segregated 2:2 with the *phoE* mutant enzyme activity.

Peptide Analysis. The specificity for immunoprecipitation of P60, P58, and P56 by APase antibody suggested that the glycosylated enzyme consists of multiple polypeptides that may or may not be antigenically and structurally related. To explore the structural relationships of the three cell-free synthesized proteins we subjected them to proteolytic fragmentation with *S. aureus* V8 protease or trypsin and analyzed the resulting peptides by electrophoretic fractionation or by HPLC. Limited hydrolysis with *S. aureus* V8 protease was accomplished by two-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis in which the first dimension was used to fractionate P60, P58, and P56 and the second dimension was a standard Cleveland gel (14) in which the P60/P56 gel region was electrophoresed longitudinally in the presence of protease. Partial digest fragments were detected by fluorography.

A large number of peptide fragments from these proteins, even at larger molecular weights, comigrated, although the kinetics of their appearance varies in some cases (Fig. 3*A*). In addition to these similarities, several predominant fragments (see arrows) appeared to be related kinetically by a single, readily susceptible peptide bond but had slightly different molecular weight from each protein. The data suggest a significant sequence homology.

HPLC of smaller peptides from more completely digested proteins revealed even more marked similarities. At least 22 of 27 [^{35}S]methionine-labeled peptides were present in all three proteins. Digestion to completion with trypsin and HPLC of the tryptic peptides yielded the profiles shown in Fig. 3*B*. Consistent with the partial hydrolyses, tryptic peptide similarities do exist. Moreover, both P58 and P56 had unique peptides not present in P60. Although only the [^{35}S]methionine-labeled tryptic peptides have been detected, these should include the NH_2-terminal tryptic peptide, because *in vitro* synthesized proteins usually retain their initiator methionine (17). If so, since P60 contains no radiolabeled peptide not found in either P58 or P56, and because it is the largest, these data suggest differences other than at the NH_2 terminus in these three related proteins.

RNA Fractionation and Sizing of Repressible APase mRNA. To establish whether the multiple repressible APase polypeptides are encoded by three monocistronic mRNAs or by a single polycistronic message, we fractionated the mRNA by preparative agarose gel electrophoresis with methyl mercury hydroxide and analyzed the size-fractionated RNA for cell-free APase mRNA activity. Translational activities for these three polypeptides were resolved into electrophoretically distinct mRNA species, indicating that separate mRNAs are responsible for their cell-free expression (Fig. 4). Their relative mobilities establish an inverse relationship between mRNA size and the polypeptides they encode.

Deglycosylation of the Native Enzyme. Previous reports on the structure of repressible APase indicate that it is a glycoprotein of approximately 300,000 daltons containing 50% mannan and 4% glucosamine (18); the protein moiety is about 60,000 daltons (19), which approximates the size of the polypeptides synthesized *in vitro*. Our results suggest that there may be more than one form of the native enzyme or that the molecule may consist of a mixture of nonidentical subunits. We therefore compared deglycosylated enzyme directly to the three *in vitro* translation products by electrophoresing endoglycosidase H-treated radiolabeled enzyme separately or mixed

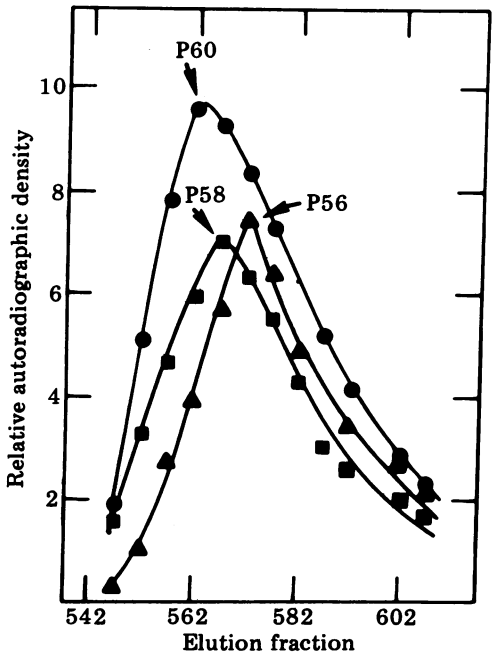

FIG. 4. Electrophoretic fractionation of APase mRNAs. Poly(A)-containing RNA from derepressed cells of strain P28-24C was fractionated by continuous-elution agarose gel electrophoresis in the presence of methyl mercury hydroxide. APase mRNA activities (P60, ●; P58, ■; and P56, ▲) were measured by gel autoradiographic assay of their immunoprecipitated cell-free translation products by densitometry.

with the cell-free synthesized proteins. The deglycosylated enzyme consisted of multiple protein bands, which we have designated D1–D4 (Fig. 5). It seems unlikely that these are

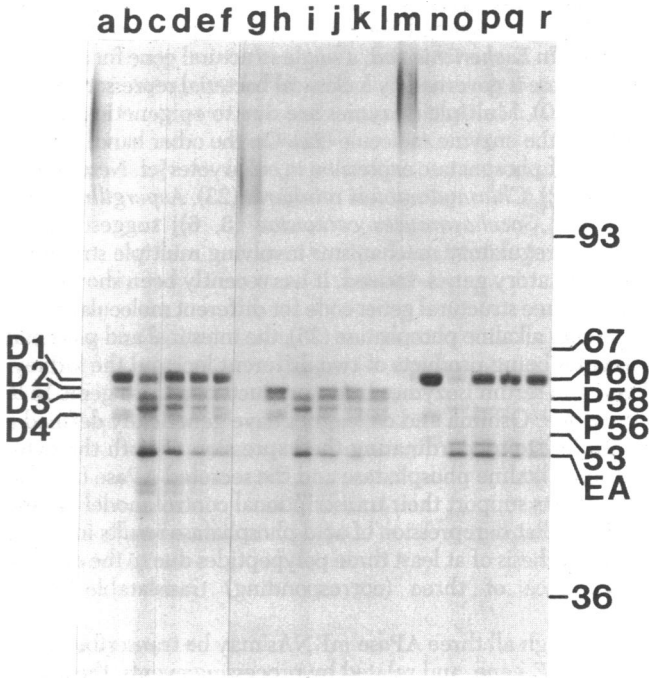

FIG. 5. Composite gel autoradiogram of deglycosylated enzyme and cell-free synthesized proteins. Lanes a–f are strain P28-24C: a, native enzyme; b, deglycosylated enzyme; c, immunoprecipitated cell-free synthesized proteins; d, 2:1 mixture of b and c; e, 4:1 mixture; f, 8:1 mixture. Lanes g–l are strain P142-4A: g, native enzyme; h, deglycosylated enzyme; i, immunoprecipitated cell-free synthesized proteins; j, 1:1 mixture of h and i; k, 2:1 mixture; l, 4:1 mixture. Lanes m–r are strain A430: m, native enzyme; n, deglycosylated enzyme; o, immunoprecipitated cell-free synthesized proteins; p, 1:2 mixture mixture of n and o; q, 1:1 mixture; and r, 2:1 mixture.

products of incomplete digestion or of contaminating protease on several grounds: no carbohydrate was detectable by a sensitive staining procedure, these protein bands were reproducibly seen in digestions with reduced amounts of enzyme, and parallel control digestions with invertase yielded single, sharp protein bands. Furthermore, four similar protein bands were observed when APase was deglycosylated by anhydrous reduction with HF (data not shown). D1 and D3 comigrated with P60 and P58, respectively. To determine if these are the products of P60 and P58 mRNA, we analyzed deglycosylated enzyme from a *phoE* mutant strain that lacks P60 mRNA activity (P142-4A) and from a strain that lacks P58 mRNA activity (A430). Shown in Fig. 5, deglycosylated enzyme from these strains also lacked D1 and D3, respectively, strongly indicating that P60 and P58 mRNAs are responsible for their synthesis. In addition, the enzyme from strain A430 lacked D2, suggesting the involvement of P58 mRNA in its synthesis. Without more information on D4 we can only speculate that it is a processed form of the primary translation product of P56 mRNA. Because the residual specific activities of the modified enzymes that were analyzed approximate the wild type, these data suggest that one or all of the polypeptides remaining have enzyme active sites and thus represent different monomers of the enzyme. The existence of three mRNAs coding for three different proteins *in vitro*, and the appearance of multiple polypeptides in the native enzyme preparation suggest that the primary translation products of these mRNAs are modified and glycosylated *in vivo* in the formation of one or more secreted glycoprotein molecules.

DISCUSSION

Most organisms possess phosphatases in multiple molecular forms which differ in structure, catalytic properties, and subcellular or tissue location. Generally, at least one form of the enzyme is glycosylated and either located periplasmically or secreted. In *Escherichia coli*, a single structural gene for alkaline phosphatase is governed by a classical bacterial repressor control circuit (20). Multiple isozymes are due to epigenetic modification of the enzyme molecule (21). On the other hand, genetic analysis of phosphatase expression in eukaryotes [cf. *Neurospora crassa* (22), *Chlamydomonas reinhardi* (23), *Aspergillus niger* (24), and *Saccharomyces cerevisiae* (3, 6)] suggests more complex regulatory mechanisms involving multiple structural and regulatory genes. Indeed, it has recently been shown that at least three structural genes code for different molecular forms of human alkaline phosphatase (25), the intestinal and placental enzymes being products of two different loci and the kidney, liver, and serum isozymes being products of a third gene. In *S. cerevisiae*, Oshima and colleagues have genetically defined a control system coordinating the expression of both the cytoplasmic alkaline phosphatase and the secreted APase (3, 4, 6). Our results support their transcriptional control model (4) and indicate that derepression of acid phosphatase results in the *de novo* synthesis of at least three polypeptides due to the *de novo* appearance of three (corresponding) translatable APase mRNAs.

Although all three APase mRNAs may be transcribed from the *PHOE* gene, and related by processing events, the ability to obtain presumptive chain termination mutants affecting the product of a specific mRNA argues for the expression of more than one structural gene. Recently, several low-P_i-induced genes have been characterized by molecular analysis of the yeast genome (R. Kramer and N. Andersen, personal communication). At least two of these hybridize to these mRNAs (unpublished results). That there are three mRNAs coding for three structurally related polypeptides is reminiscent of the ovalbumin system in which the ovalbumin gene is flanked by two related genes (*X* and *Y*). These have similar intron and exon patterns containing related exon sequences that code for three cellular mRNAs (26). The products of the APase mRNAs which are expressed *in vivo* may simply exist as multiple isoenzymes, although it is possible that these polypeptides are different subunits of a heterologous oligomeric protein. This would imply an evolutionarily well-developed gene system for such a protein complex. On the other hand, spontaneous gene duplications of APase in *S. cerevisiae* have been measured experimentally under forced growth conditions and occur at frequencies between 10^{-11} and 10^{-12} duplications per mitosis (27). In either case, the coordinate differential expression and glycosylation of these polypeptides are features of a system proving to be excellent for studying the cellular mechanisms coordinating gene expression, glycosylation, and secretion of the enzyme.

We thank Drs. Akio Toh-e and Yasuji Oshima for their generous provision of information, strains, and preprints, Dr. P. W. Robbins for endogylcosidase H, Drs. M. E. Schweingruber, David Rogers, and Daniel Perlman for information and comments, and Ms. Rosalind Lee and Ms. Teresa Willcocks for technical assistance. This work was supported in part by U.S. Public Health Service Grant AI 1060 (H.O.H.) and by an American Cancer Society Postdoctoral Fellowship (K.A.B.).

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