Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2157–2161, April 1982
Biochemistry

Acid phosphatase polypeptides in *Saccharomyces cerevisiae* are encoded by a differentially regulated multigene family

(transcriptional regulation/yeast transformation/hybridization-selection/molecular evolution/yeast promoter)

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Communicated by Martin Gibbs, December 30, 1981

ABSTRACT **Two clones from a λ phage collection containing yeast genes regulated by inorganic phosphate were shown by low-stringency hybridization to select three mRNAs that direct the *in vitro* synthesis of repressible acid phosphatase (EC 3.1.3.2) polypeptides p60, p58, and p56. By higher stringency hybridization one yeast fragment [8 kilobases (kb)] selects p60 mRNA and the other (5 kb) selects p56 mRNA. These *Eco*RI digestion fragments were subcloned in yeast transformation vectors and hybridization selection assignments were confirmed by measuring enzyme and mRNA levels in transformants. Enzyme and mRNA levels in (8-kb) high copy number transformants grown in high inorganic phosphate medium revealed a hitherto undetected acid phosphatase protein, P57, which is believed to correspond to the constitutive enzyme encoded by *PHO3*. The identity of the 8-kb fragment purported to contain the *PHO5/PHO3* genes was confirmed by genetic mapping of an integrated copy of this fragment. The site of integration of the 5-kb fragment was demonstrated to be unlinked to the *PHO5/PHO3* genes.**

In *Saccharomyces cerevisiae* a complex genetic system controls the expression of a number of phosphohydrolases in response to cellular levels of inorganic phosphate (P_i) (1–4). Included in these enzymes is a cytoplasmic repressible alkaline phosphatase (EC 3.1.3.1), a repressible acid phosphatase [APase; orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2], and an acid phosphatase synthesized during high-P_i growth. Structural genes for these enzymes are *PHO8*, *PHO5*, and *PHO3*, respectively (2, 5, 6). The repressible acid phosphatase is an exocellular, multimeric glycoprotein with an average mass of 490 kilodaltons (7, 8), consisting of several sequence-related polypeptides. These variant polypeptides, probably forming different isoenzymes in a heterologous quaternary structure, are encoded by three distinct mRNAs (9), distinguishable by their antigenic products in cell-free translation (p60, p58, and p56). We previously established that expression of these mRNAs is regulated by P_i and that repressible APase is synthesized *de novo* upon derepression (9), consistent with a transcriptional regulation model proposed by Oshima and colleagues (10). According to their genetic model, *PHO5* and *PHO8* are transcriptionally regulated by a coordinate mechanism involving the products of numerous regulatory genes that interact at the posttranslational level. *PHO3* expression is mediated by two genes independent of this mechanism.

Recently, several yeast genes under P_i control were isolated by Kramer and Andersen (11). Two of these genes, defined by *Eco*RI restriction endonuclease fragments of 8 and 5 kilobases (kb), are regulated in a fashion characteristic of phosphatase structural genes. We have established the identity and chromosome location of these transcriptionally regulated genes, using yeast transformation and hybridization selection. We demonstrate here that the 8-kb *Eco*RI fragment contains two phosphatase structural genes, one encoding p60 and another encoding a previously unidentified antigenic polypeptide, p57. Genetic and biochemical data identify p60 as the gene product of *PHO5* and p57 as the product of the genetically linked *PHO3* gene. The 5-kb *Eco*RI fragment contains another phosphatase gene, unlinked to the *PHO5 PHO3* loci, encoding p56. On the basis of hybridization data the p58 mRNA is postulated to be the product of a third physically and genetically undefined gene. These phosphatase genes presumably arose by duplication of an ancestral gene, and are all expressed. A unique and interesting observation from this work is that the *PHO3* and *PHO5* genes through duplication and subsequent evolutionary divergence have become regulated by genetically distinct and disparate mechanisms.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media. Yeast strains H42 (*a gal4*), P28-24C (*a pho3*), and P142-4A (*α arg pho3 pho5*) were kindly provided by A. Toh-e and Y. Oshima (Osaka University, Osaka, Japan). DB745 (*α ade1 leu2 ura3*) and DB689 (*a leu2 ura3 can1*) were from D. Botstein (Massachusetts Institute of Technology, Cambridge, MA). S288C (*α gal2 mal suc2*), DR1134B (*a ura3 lys2*), DR1134C (*α ura3 lys2*), and DB4 (*α pho5 ura3*) were from our stock collection. High-P_i or low-P_i YEPD and SMD media were as described (9). UMD media were Burkholder minimal medium (9) containing an amino acid mixture plus adenine but lacking uracil, and containing either KH_2PO_4 at 0.3 g/liter (high-P_i) or KCl at 0.3 g/liter and KH_2PO_4 at 0.03 g/liter (low-P_i).

Enzyme Activity. APase activity in whole cells was assayed with *p*-nitrophenyl phosphate as substrate (9). One unit of enzyme is that amount producing 1 μmol of *p*-nitrophenol per min. Activity is expressed relative to the culture density measured by turbidity at 660 nm.

Recombinant Phage. The recombinant phage λYlp8, λYlp18, λYlp20, and λYlp23 were isolated by Kramer and Andersen (11) from a partial *Eco*RI S288C DNA bank. The yeast DNA fragments from these phage, obtained by agarose gel electrophoresis, were kindly provided by R. Kramer (National Institutes of Health, Bethesda, MD).

Construction of Recombinant Plasmids. All restriction enzymes and phage T4 DNA ligase were from New England BioLabs; these enzymes were used according to the supplier's specifications. Recombinant plasmids were constructed by using *Eco*RI-digested DNA from the recombinant phage and the plasmid YEp24 (12). The mixture of fragments was ligated and

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Abbreviations: APase, acid phosphatase; kb, kilobase(s).

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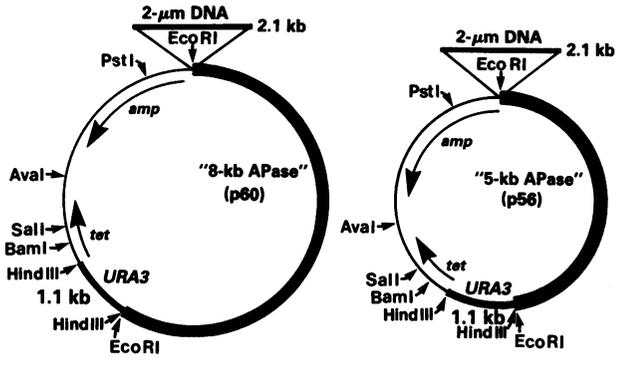

FIG. 1. Restriction maps of phosphatase plasmids.

the products were used to transform *Escherichia coli*. Four ampicillin-resistant clones were identified, which carry plasmids containing the 8-kb fragment from λYlp20, with (YEpAP11) and without (YIpAP11) the 2-μm fragment or the 5-kb fragment from YIP18, with (YEpAP31) and without (YIpAP31) the 2-μm fragment (Fig. 1).

Cell Growth and Nucleic Acid Preparation. DNA was prepared by the method of Hereford and Rosbash (13). Total cellular RNAs were prepared as described (9) from yeast cultures started with fresh overnight innocula diluted to 10^6 cells per ml into low-P_i or high-P_i SMD medium. Transformed yeast strains were grown in UMD media. Enzyme assays and RNA preparations were made from the same culture harvested at a density of 3.75×10^7 cells per ml. This gives 50% of the maximal derepressed level of APase activity for wild-type strain H42.

Cell-Free Protein Synthesis and Immunoprecipitation. RNAs were translated in a wheat-germ cell-free system using L-[^{35}S]methionine (9). Specific cell-free translation products were analyzed by immunoprecipitation, electrophoresis, and fluorography, according to published methods (9, 14). Synthetic yield for APase and several other yeast proteins, determined by autoradiographic densitometry, was proportional to the concentration of their specific mRNA (unpublished data). Immunocompetition assays were previously described (9, 14).

Hybridization Selection. RNAs complementary to recombinant phage and plasmid clones were selected from total cellular RNAs by hybridization either under R-loop conditions (15), or by solid-state hybridization to DNA covalently attached to diazobenzyloxymethyl paper (16). R-loop hybrids were isolated chromatographically and denatured by heat treatment (100°C, 1 min). Solid-state hybridization filters were stringently washed at 50°C in 50% (vol/vol) formamide/5 mM Tris·HCl, pH 7.5/15 mM NaCl/0.1 mM EDTA/0.1% $NaDodSO_4$ prior to elution of bound RNA. Selected RNAs were identified by *in vitro* translation.

Yeast Transformation and Genetic Analysis. Yeast transformation was by the method of Hinnen *et al.* (17) and transformants were selected by auxotrophic complementation on uracil-deficient medium. Transformed clones were screened for stable transformants by testing for reversion to uracil auxotrophy upon subcloning on complete medium. Genetic analysis was by standard methods (9).

Southern Blot Analysis. DNA was digested with *Xho* I, electrophoresed on 0.5% agarose gels, transferred to nitrocellulose, and probed by hybridization with ^{32}P-labeled nick-translated plasmid DNA (18, 19).

RESULTS

Analysis of Recombinant Phage by mRNA Selection and Translation. Previously, we demonstrated the repressible synthesis of APase by immunoprecipitation of [^{35}S]methionine-labeled cell extracts with antibody to the native glycoprotein (9). We identified and correlated four *in vivo* proteins (D1–D4) with the *in vitro* translation products of three distinct APase mRNAs (p60, p58, and p56). On the basis of the peptide maps of these proteins and the ability to obtain chain termination mutations affecting the product of a specific mRNA, we postulated the existence of more than one structural gene (9).

The isolation by Kramer and Andersen (11) of several genes induced under low-P_i conditions allowed us to examine this postulate. RNAs were selected by hybridization to these genes and translated *in vitro*, and APase specific translation products were identified by immunoprecipitation. Initially, total cellular RNA from strain P28-24C grown in low-P_i medium was hybridized to DNA from phages λYlp8, -18, -20, and -23 under R-loop hybridization conditions, and the hybrids were isolated by exclusion chromatography. Column fractions were translated with or without prior heat denaturation of the RNA·DNA hybrids, and the immunoprecipitated reaction products were analyzed by electrophoresis and fluorography (Fig. 2*A*). Two phage, λYlp20 and λYlp18, showed identical hybridizations to all three APase mRNAs. The mRNA activity was fully detectable in the column void volume only after denaturation (Fig. 2*A*, lanes g–i), authenticating the formation of RNA·DNA hybrids. In both cases, enolase mRNA, assayed as an internal control, eluted in the included volume of the column and was unaffected by the heat treatment (Fig. 2*A*, lanes d–f and j–l). DNA from λYlp8 and λYlp23 did not select mRNAs with detectable *in vitro*

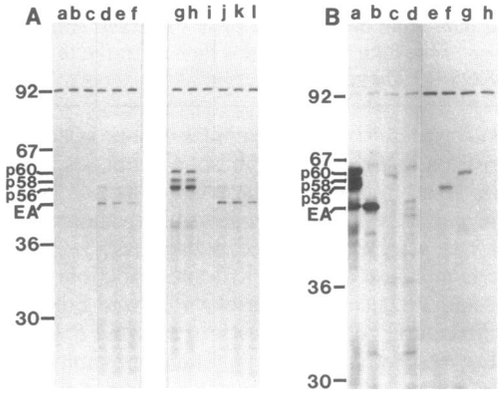

FIG. 2. Identification of genes induced under low-P_i conditions. (*A*) RNA from low-P_i-grown cells of strain P28-24C was hybridized to the purified 8-kb *Eco*RI fragment of phage λYlp20 under conditions favoring R-loop formation and passed through a Bio-Gel A-150m column. Three fractions from the included and void volume of the column were collected. Heat-treated and untreated portions of each fraction were used to program cell-free translations in a mixture containing [^{35}S]methionine. Translation products were immunoprecipitated with a mixture of IgG antibodies to APase and enolase (EA) and electrophoresed on a $NaDodSO_4$/10% polyacrylamide gel, followed by autoradiography. Positions of markers of 92, 67, 36, and 30 kilodaltons are shown on the left. [^{14}C]Phosphorylase B, added to the samples as a norm, migrated at the 92-kilodalton marker. Lanes a–c are void-volume and lanes d–f are included-volume fractions untreated prior to translation; lanes g–i are void-volume and lanes j–l are included-volume fractions heat-treated prior to translation. Similar results were obtained with the purified 5-kb *Eco*RI fragment of λYlp18. (*B*) The yeast *Eco*RI fragments contained in λYlp8, -18, -20, and -23 were immobilized on diazobenzyloxymethyl paper and hybridized with RNA from low-P_i-grown cells of strain P28-24C. Hybridized RNAs were eluted by heat treatment and translated. Lanes a and b are the translation products of RNA from derepressed (a) and repressed (b) cells immunoprecipitated with a mixture of IgG antibodies to APase and enolase. Lanes c and d are preimmune IgG precipitations of the same samples as a and b. Lanes e–h are the translation products of RNA selectively hybridized to the DNA from λYlp8 (lane e), λYlp18 (lane f), λYlp20 (lane g), and λYlp23 (lane h) immunoprecipitated with a mixture of IgG antibodies to APase and enolase.

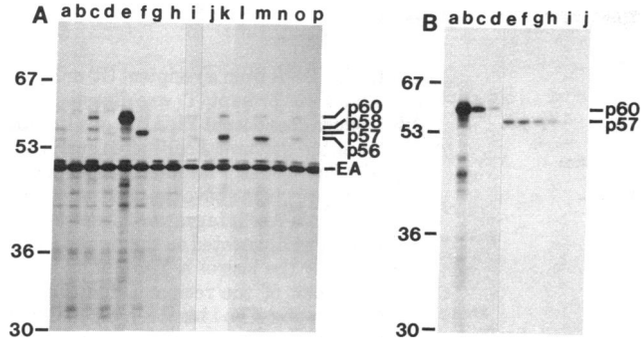

FIG. 3. Expression of APase mRNA in transformed yeast carrying the 8-kb and 5-kb phosphatase plasmids. RNA was purified from repressed and derepressed cells of the recipient strains and transformants carrying the phosphatase plasmids shown in Fig. 1. RNA was assayed by the procedures described for Fig. 2. Positions of markers of 67, 53, 36, and 30 kilodaltons are shown on the left. (*A*) Immunoprecipitated translation products of RNA from derepressed and repressed cells, respectively, of DB4 (lanes a and b), DB4-YIpAP11 (lanes c and d), DB4-YEpAP11 (lanes e and f), DB4-YEpAP11 revertant (lanes g and h), DB745 (lanes i and j), DB745-YIpAP31 (lanes k and l), DB745-YEpAP31 (lanes m and n), and DB745-YEpAP31 revertant (lanes o and p). (*B*) Translation products of RNA from derepressed cells of DB4-YEpAP11 immunoprecipitated with APase IgG plus 0 (lane b), 0.5 (lane c), or 1 (lane d) μg of nonradioactive APase; or from repressed cells immunoprecipitated with APase IgG plus 0 (lane e), 10 (lane f), 25 (lane g), 55 (lane h), 100 (lane i), or 250 (lane j) ng of APase; or precipitated with preimmune IgG (lane a).

activity and were not studied. Because the peptide maps of p60, p58, and p56 suggested that the mRNAs encoding these proteins would have considerable sequence homology, we anticipated the hybridization of all three phosphatase mRNAs to more than one DNA fragment under low-stringency hybridization conditions. RNA complementarity was therefore also determined by a more stringent solid-state hybridization selection procedure (Fig. 2*B*). By this method of selection and identification, the 5-kb *Eco*RI fragment of phage λYlp18 hybridizes specifically with p56 mRNA (Fig. 2*B*, lane f) and the 8-kb *Eco*RI fragment of phage λYlp20 hybridizes specifically with p60 mRNA (Fig. 2*B*, lane g). We have thus assigned these two mRNAs as transcripts of two distinct phosphatase genes.

***In Vivo* Expression of the Cloned Phosphatase Genes After Yeast Transformation.** Confirmation of the above gene assignments was made by determining the *in vivo* biological activity of the 5-kb and 8-kb *Eco*RI fragments. The *Eco*RI fragments were subcloned from λ into yeast transformation vectors that contained the yeast-selectable *URA3* gene, with or without an origin of replication from the yeast 2-μm plasmid (Fig. 1). The recipient transformation strain for plasmids YIpAP11 and YEpAP11, which contain the 8-kb *Eco*RI fragment, was DB4, a *ura3 pho5* segregant from a cross of DB689 and P142-4A. This strain was used because of its failure to produce functional p60 mRNA (Fig. 3*A*, lanes a and b) and APase activity was 10% of that in wild-type strain H42. Transformants carrying YIpAP11 or YEpAP11 plasmids were selected by uracil prototrophy. Revertants that had lost YEpAP11 were identified and isolated after growth on nonselective medium. These strains were analyzed for enzyme activity and for APase cell-free mRNA activity. Because derepressed cells accumulate cellular enzyme activity and mRNA well into stationary phase, comparisons were made on cultures grown under the same conditions and harvested at a fixed cell density. In wild-type strain H42, APase activity increases by at least 100-fold during derepression, and when fully derepressed, the enzyme constitutes more than 1% of the total cellular protein. The integrative transformant (plasmid without 2-μm DNA) showed a 2.5-fold increase over the enzyme level of the recipient when grown in low-P_i medium and the nonintegrated transformant (multicopy plasmid containing 2-μm DNA) a 24-fold increase (Table 1), consistent with these plasmids carrying a functionally active phosphatase gene. In the YEpAP11 transformant, the *PHO5* gene dosage is 20- to 30-fold increased over its parent, matched by a 30-fold increase in gene expression. Assays for mRNA revealed the appearance, in both transformants, of p60 mRNA activity for cells grown in low-P_i medium (Fig. 3*A*, lanes c and e), which was absent in the revertant (Fig. 3*A*, lane h). The proportional level of p60 mRNA activity in the transformants (Table 1) corresponded to the proportional increase in APase activity for the derepressed cells, consistent with the p60 gene residing in the 8-kb *Eco*RI fragment. The enzyme activity of cells grown in high-P_i medium increased 2-fold in the integrative transformant and 23-fold in the self-replicating transformant. However, cells grown under these conditions showed essentially no detectable p60, p58, or p56 mRNA (Fig. 3*A*, lanes d, f, and h). Instead, cells of the recipient strain possess a low level of mRNA directing the synthesis of a 57-kilo dalton crossreactive polypeptide (p57) that is increased 2-fold in the integrative transformant and 24-fold in the self-replicating transformant. The p57 species is undetectable among the translation products of RNA from low-P_i-grown

Table 1. Enzyme and mRNA activities of recipient and transformed yeast strains

Strain	Enzyme activity, units/OD_{660}		*In vitro* mRNA activity, relative density units			
			High-P_i	Low-P_i		
	High-P_i	Low-P_i	p57	p60	p58	p56
H42 (wild type *PHO3 PHO5*)	0.019	0.572	—	87	76	96
DB4 ($p60^-$)	0.062	0.082	9	—	23	28
DB4-YIpAP11 (integrative)	0.118	0.209	21	57	25	29
DB4-YEpAP11 (self-replicating)	1.412	1.995	218	1847	22	22
DB4-YEpAP11 revertant	0.053	0.065	7	—	21	25
DB745 ($p58^-$)	0.049	0.204	—	30	—	40
DB745-YIpAP31 (integrative)	0.051	0.397	—	38	—	126
DB745-YIpAP31 (self-replicating)	0.057	0.433	—	25	—	142
DB745-YIpAP31 revertant	0.070	0.243	—	25	—	36

Cells were grown as described in the text and assayed for enzyme activity and translatable mRNA. *In vitro* mRNA activities were determined by densitometry of the corresponding translation product from gel autoradiograms, normalized to the corresponding activity of enolase mRNA, which remains a constant proportion of total cellular mRNA during changing cellular phosphate concentration.

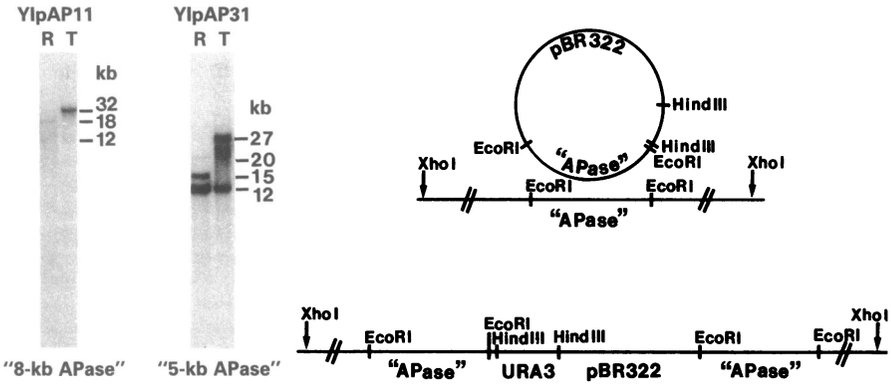

FIG. 4. DNA from a recipient (R) or an integrative transformant (T) was digested with *Xho* I, which does not cut YIpAP11 or YIpAP31 or within the genomic *Eco*RI sites surrounding all APase genes. Plasmids used for transformation are given above the gels; probes are given below. An integration event at an APase site, as illustrated on the right, is demonstrated by the loss of a *Xho* I band in the Southern blot of the recipient and the production of a new *Xho* I band corresponding in size to the lost recipient band plus the molecular weight of the plasmid.

cells (Fig. 3*A*, lanes a, c, e, g, i, k, m, and o). The antigenicity of this previously uncharacterized polypeptide, and the p60 polypeptide synthesized *in vitro* by RNA from transformed cells, was confirmed by an immunocompetition assay using pure APase (Fig. 3*B*). The observed parallel changes in mRNA and enzyme levels are consistent with p57 being a phosphatase encoded by a gene closely linked to the p60 gene and under reverse phosphate regulation. Two regions of close homology within the 8-kb *Eco*RI fragment have been observed by heteroduplex analysis (R. Kramer, personal communication), suggesting that p57 is the "constitutive" acid phosphatase of the *PHO3* gene shown by genetic analysis to map at the *PHO5* locus.

Transformations were also performed with the plasmids containing the 5-kb *Eco*RI fragment. Unlike the *pho5* mutants, which lack functional p60 mRNA, no yeast strain lacking p56 mRNA has been observed, and therefore, a standard high-frequency transformation strain, DB745 was used as a recipient. This strain has low levels of p60 and p56 mRNA activity (when grown in low-P_i medium) and lacks functional p58 mRNA (Fig. 3*A*, lanes i and j). The absence of p58 mRNA activity is common to many laboratory yeast strains. The integrative plasmid YIpAP31 transformed at a higher than expected frequency, comparable to that of YEpAP31, but reverted to uracil auxotrophy at a frequency of approximately 1/500, indicating that YIpAP31 carries an origin of replication. A stable integrant of YIpAP31 was obtained by repeated subcloning on rich medium and selection on uracil-deficient medium. Enzyme levels for the strains transformed with the integrative and nonintegrative plasmids containing the 5-kb fragment increased about 2- to 3-fold above the recipient. The revertant for plasmid YEpAP31, selected as above, returned to the recipient enzyme level. Translation of RNA isolated from derepressed cells showed an increase of 3-fold in the transformed cells for p56 mRNA activity and no increase in the levels of p60 (Fig. 3*A*, lanes k and m). This is consistent with p56 being encoded by a gene contained within the 5-kb *Eco*RI fragment. There were no changes in the levels of p56 or p60 mRNA activity (Fig. 3*A*, lanes l and n) or in enzyme levels in repressed cells.

Genomic Mapping of the Integrated Transformants. Genetic confirmation that *PHO5* is the p60 gene was obtained by mapping the integrated copy of *URA3* in the YIpAP11 transformant to *lys2*, which lies close to *PHO5* on chromosome II (20). The site of YIpAP11 integration was shown to be at its homologous genomic sequence by Southern gel analysis. DNA from DB4 digested with the endonuclease *Xho* I gave two bands (18 and 12 kb) when probed with labeled *Eco*RI fragments from λYlp18 and λYlp20 (Fig. 4). The 18-kb band hybridized strongly to the 8-kb fragment (p60, p57) but weakly to the 5-kb fragment (p56), whereas the 12-kb band hybridized strongly to the p56 *Eco*RI fragment and weakly to the p60, p57 fragment. In the YIpAP11 transformant, the 18-kb fragment is lost and is replaced with a 32-kb band strongly hybridizing to the p60, p57 fragment or pBR322. The plasmid therefore has integrated at its phosphatase homologous site and not at the *URA3* site or at another phosphatase homologous site. This transformed strain was crossed with the strain DR1134B (*lys2 ura3*) and the diploid was subjected to tetrad analysis (Table 2). The transformed *URA3* gene maps close to *lys2* (20 centimorgans), the expected distance for plasmid integration at *PHO5*. Thus, the p60 and p57 genes, contained within λYlp20, map to the *PHO5/PHO3* locus as diagrammed in Fig. 5.

A similar analysis was performed with the YIpAP31 integrative transformant, containing the p56 gene. Southern gel analysis of DNA from strain DB745 digested with endonuclease *Xho* I gave three bands hybridizing to a p56 *Eco*RI probe (Fig. 4). The largest band (20 kb) hybridized strongly to the p60 fragment and weakly to the p56 fragment and therefore contained the *PHO5* gene. The second (15-kb) and third (12-kb) fragments hybridized strongly to the p56 fragment. In the YIpAP31 transformant the intermediate 15-kb fragment is lost and replaced by a 27-kb band hybridizing to the p56 *Eco*RI fragment or pBR322, consistent with the integration of YIpAP31 within the 15-kb fragment and at a site distinct from the *PHO5* gene. Genetic analysis of this transformant (Table 2) showed that the integrated *URA3* gene was not linked to *lys2*, confirming that there is at least one site unlinked to *PHO5* but having close

Table 2. Genomic mapping of integrated APase plasmids

Loci	Non-parental ditype	Parental ditype	Tetra-type	Linkage, centi-morgans
8-kb fragment (p60, p57)				
URA3-LYS2	0	17	12	20
5-kb fragment (p56)				
URA3-LYS2	5	10	24	Unlinked
URA3-LEU2	2	5	32	Unlinked

Strains transformed with YIpAP11 and YIpAP31 were crossed to a *lys2 ura3* strain and the diploids were subjected to tetrad analysis. The frequency of segregation patterns and the linkage are tabulated.

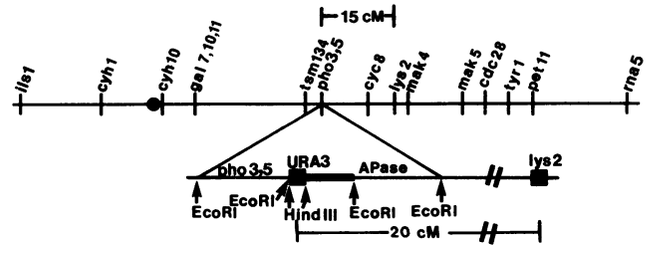

FIG. 5. Genomic map of chromosome II and comparison of the map distance of *URA3* and *lys2* in the integrative transformant for the 8-kb *Eco*RI fragment. cM, Centimorgan.

homology to it in the yeast genome. The frequency of asci that were tetratype with respect to the integrated *URA3* and the centromere-linked marker *leu2* (Table 2) indicated that the site of integration is not centromere linked.

DISCUSSION

Although it has previously been demonstrated that repressible APase in *S. cerevisiae* consists of several sequence-related polypeptides synthesized from three distinct mRNAs (9), only two APase structural genes have been identified genetically (2, 5), and only one of these (*PHO5*) encodes a repressible enzyme, whereas the other (*PHO3*) encodes an enzyme reportedly synthesized constitutively. Our objective in this paper was to analyze a collection of cloned yeast genes exhibiting the requisite properties of APase structural genes in order to identify and establish their relationship to the three mRNAs and to the *PHO3 PHO5* locus. Our data has led us to the conclusion that yeast acid phosphatase is encoded in a family of at least four expressed genes. All three APase mRNAs selectively hybridized to two of the cloned genes under low stringency, confirming their extensive homology. From more stringent hybridizations with these genes and from their expression *in vivo*, we have established that p60 and p56 are the unique products of two distinct genes. Genetic mapping of these genes, integrated genomically, demonstrates that the *PHO5* locus encodes p60 and that the p56 gene is unlinked to *PHO5*. Our inability to selectively hybridize p58 mRNA to either gene under stringent conditions or to effect its expression *in vivo* by transformation suggests that it is transcribed from a third gene yet to be identified.

Our analysis of the 8-kb *Eco*RI fragment revealed the presence of a second gene on this fragment (*PHO3*) in addition to, and contiguous with, *PHO5*. The product of this gene is the previously unidentified protein p57. This conclusion is based on the genetic linkage of *PHO5* and *PHO3*, the regulated expression of the p57 gene, and the comparable antigenicity of p57 to the APase polypeptides. All of our data indicate that *PHO3* is induced by P_i and expressed only when cells are grown in high-P_i medium, which is a reverse regulation to that of *PHO5*. The proximity of these tandem, related genes suggests that *PHO5* and *PHO3* arose by duplication of an ancestral gene. This duplication must have been followed by a divergence in the modes of regulation of the two genes. Moreover, the reverse regulation of *PHO3* by P_i involves at least two regulatory genes that do not participate in the expression of *PHO5* (20). However, at least one regulatory gene may participate in the expression of both *PHO3* and *PHO5*. In experiments not presented here we have established that transcription of the *PHO3* gene (p57) in high-P_i-grown cells is affected by a mutation in *PHO80* that causes constitutive synthesis of the *PHO5* gene product. This constitutive, conditional regulatory mutant allows the expression of *PHO5* but not *PHO3* at the nonpermissive temperature. This observation may imply a direct role for *PHO80* in *PHO3* expression and a coordinate mechanism for the reverse regulation of *PHO3* and *PHO5*. Alternatively, this may simply be due to a passive mechanism involving *PHO5* transcription.

Our results reveal an extremely tight regulation of the *PHO3* and *PHO5* genes at high dosage in strains carrying the self-replicating plasmid YEpAP11. A 20- to 30-fold increase in gene dosage resulted in a similar increase in gene expression, but no alteration was observed in their regulation by P_i. This may have implications for the mechanism of regulation. If the regulated expression of these genes involves the direct participation of a positive effector of transcription, as has been proposed for *PHO5* by Toh-e *et al.* (10), there must be as many molecules of the regulatory factors as structural genes. For the galactose utilization pathway enzymes a similar model has been proposed (21, 22); in this model the positive effector, encoded by *GAL4*, has been shown to exhibit a gene dosage effect on uridine diphosphogalactose-4-epimerase activity in the tetraploid strain (*GAL4, gal4, gal4, gal4*), suggesting 4 or 5 copies of the *GAL4* product per gene copy (23). This means that single-copy phosphatase regulatory genes encode interactive regulatory elements 6-fold above the level of *GAL4* expression, or multiple copies of the regulatory genes exist, or a mechanism other than direct interaction is responsible for regulating the expression of *PHO5*. A similar argument can be made for the regulatory factors involved in expression of *PHO3*.

The origin and significance of the yeast APase multiple gene family remains to be determined. Information available from peptide mapping and nucleic acid hybridization suggests that the structural genes for the three APase loci repressed by P_i have undergone considerable divergence, while all three loci remain under strict and identical regulation. A comparison of the promoter regions of these genes may reveal conserved sequences important for their strict and coordinate regulation.

We thank R. Kramer and colleagues for providing us with recombinant phage DNA from their isolates, and K. Turner, R. Kramer, G. Thill, T. Barnett, and J. Woolford for helpful information and discussion. This research was conducted in the laboratory of Dr. H. O. Halvorson and was supported in part by U.S. Public Health Service Grant AI10610. D.T.R. was a Charles A. King Trust Research Fellow.

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