JOURNAL OF BACTERIOLOGY, Nov. 1974, p. 608-617

Vol. 120, No. 2
Printed in U.S.A.

Characterization of a Dominant, Constitutive Mutation, *PHOO*, for the Repressible Acid Phosphatase Synthesis in *Saccharomyces cerevisiae*

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Received for publication 20 August 1974

An apparent operator-constitutive mutation was discovered in the repressible acid phosphatase system in *Saccharomyces cerevisiae*. The site of mutation, designated *PHOO*, was found to be closely linked to the *phoD* locus. The mutant allele, *PHOO*, was semidominant over the wild-type allele and effective for the expression of the *phoD* gene in *cis* position. The *phoD* mutation gave rise to a defective phenotype for the formation of the repressible acid phosphatase. On the other hand, neither the repressible acid phosphatase activity in the cell-free extracts prepared from cells of the temperature-sensitive *phoD* mutant grown at 25 C, nor that of the revertants from the *phoD* mutants, could be distinguished from that of the wild-type strain with respect to thermolability and K_m value for *p*-nitrophenylphosphate. These results strongly suggest that the *phoD* gene is not a structural gene, but a regulatory gene exerting positive control for the formation of repressible acid phosphatase. Close similarity between the apparent role of the *phoO-PHOD* gene cluster and that of the *c-GAL4* gene cluster in the galactose system of *S. cerevisiae* could be inferred.

Derepression of the synthesis of nonspecific phosphatases (EC 3.1.3.1 and EC 3.1.3.2) under conditions of low orthophosphate concentration has been described in several microorganisms, both prokaryotic (13, 18) and eukaryotic (2, 19, 20, 22, 24). In *Escherichia coli*, two separate gene loci are involved in the regulation of the alkaline phosphatase system (6). One of these loci, *phoR* (R1), was assumed to play an essential role in the formation of the enzyme (9, 10). The other, *phoS* (R2), from which two classes of constitutive mutations were obtained, was suggested to be primarily involved in transport of inorganic phosphate (28). However, there is no evidence at present bearing on which site recognizes the regulatory factor. In a comparative study of the mechanism of phosphatase synthesis operating in prokaryotes and eukaryotes under similar physiological conditions, the repressible acid phosphatase system of *Saccharomyces cerevisiae* is of particular interest, because of the ease of isolation of mutants by the diazo coupling method described by Dorn (3), and because of the convenience of performing genetic and biochemical studies on this organism. Schurr and Yagil (23) have described mutants that lacked the acid and alkaline phosphatase activities or that showed the constitutive formation of the enzymes.

In a previous study (26), we isolated many acid phosphatase mutants of *S. cerevisiae*. Genetic and biochemical analyses of these mutants revealed the existence of two species of acid phosphatase, one that is constitutive and another that is repressible by inorganic phosphate. The formation of the constitutive enzyme is controlled by the *phoC* gene on chromosome II, whereas that of the repressible one is controlled by the *phoB*, *phoD*, *phoE*, *phoR*, and *phoS* genes, as well as by the concentration of inorganic phosphate in the medium. Of these, *phoR* and *phoS* have clearly been indicated to be the regulatory genes controlling the formation of the repressible enzyme. The *phoE* gene, which is closely linked to the *phoC* gene, is most probably the structural gene for the repressible enzyme (to be published elsewhere). The fact that the *phoD* mutation gives rise to the absence of both repressible acid and alkaline phosphatase activities suggests that the *phoD* gene might produce a common precursor for both the enzymes, or that it might have a positive regulatory function of derepressing these phosphatases. Since the *phoR* gene seems to exert a negative control over the formation of the repressible phosphatases by coding for the repressor molecule, it was expected that a receptor site for the product of the *phoR* gene

would be found. A mutation occurring in such a site could result in a phenotype similar to the operator-constitutive mutation in bacterial systems. Such a mutation has indeed been found, and preliminary characterization of the mutant has been described (26). This paper deals with the genetic characterization of the dominant constitutive mutations for the acid phosphatase formation. One of the mutations was found to be an operator-constitutive type at a site contiguous to the *phoD* locus.

MATERIALS AND METHODS

Organisms and media. The strains of *S. cerevisiae* employed in this study were derivatives of strains used in the previous study (26). All of them carried the *phoC-1* mutant allele to eliminate constitutive acid phosphatase activity, unless otherwise noted. Composition of the culture media in high-P_i (1,500 mg of KH_2PO_4 per liter in modified Burkholder synthetic medium), low-P_i (30 mg of KH_2PO_4 and 1,500 mg of KCl per liter of the same medium), nutrient, and sporulation media were as described previously (26).

Isolation of mutants. In addition to the mutant previously isolated by ultraviolet irradition (26), several new mutants for the acid phosphatase production were collected after mutagenesis with ethyl methane sulfonate (EMS) by the method of Lindegren et al. (15). Mutants of similar phenotypes were obtained in roughly the same frequencies by either EMS or ultraviolet mutagenesis. Each mutant was isolated from a culture grown at 35 C and was tested for its ability to produce the acid phosphatase at 25 and 35 C.

Genetic analysis. The general techniques for yeast genetics described previously (26) were followed throughout in this study.

Detection and determination of the acid phosphatase activity. The acid phosphatase activity of colonies growing on plates was detected by staining (3, 26). Colonies were overlaid with molten soft agar containing 0.5 mg of α-naphthylphosphate per ml, 5 mg of Fast blue salt B per ml, and 1% agar in 0.05 M acetate buffer (pH 4.0). (Concentration of the staining reagents were diluted to one-tenth of the original formula [26]. No inconvenience was encountered with the new formula.) The acid phosphatase activity was assayed by using intact-cell suspensions or cell extracts as the enzyme source and *p*-nitrophenylphosphate as the substrate. The detailed methods for cultivation of cells, preparation of extracts with a Braun cell homogenizer, and assay of the enzyme activity have been described previously (26). Each extract used in this study was dialyzed against 0.01 M acetate buffer (pH 4.0) at 0 C overnight. One unit of enzyme was defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per min at 35 C.

RESULTS

Isolation of dominant constitutive mutants. For the isolation of the constitutive mutants for the acid phosphatase formation, cells of the *phoC-1* strains, such as strain P-22-14D (α) and P-28-24C (a), were plated on high-P_i medium after mutagenic treatment with EMS. Colonies determined to be constitutive for phosphatase synthesis by staining were isolated. A constitutive mutant, O27-M66, that was isolated in the previous study by ultraviolet irradiation (26), and its haploid derivatives, strains OC-6A and P-76-4B having the α and a mating-type, respectively, were included for further study. These two derivatives were selected from the tetrad segregants of crosses between strain O27-M66 and the *phoC-1* strains, and their genotypes were confirmed to be the same as strain O27-M66 with respect to the repressible acid phosphatase formation from the genetic results.

Each of these mutants was crossed to the *phoC-1* strains. The resulting diploids were tested, by the staining method, for their ability to produce the acid phosphatase on high-P_i medium. Mutants that showed significant acid phosphatase activity as diploids were collected. Since the acid phosphatase was produced more efficiently in nutrient medium than in high-P_i medium, nutrient broth was used in place of high-P_i medium to examine enzyme formation in repressed condition. The dominant constitutive mutants and the diploid heterozygotes were grown in nutrient and low-P_i media at 30 C for 24 h with shaking. A *phoR* mutant, P-32-2B, and a diploid prepared by crossing it to the *phoC-1* strain, P-28-24C, were also grown as above. Samples of the culture were taken at appropriate intervals. Cells were washed once with distilled water and were suspended in distilled water to the original volume. This suspension of intact cells was used as the enzyme source for assay of the acid phosphatase activity. The maximal specific activities attained during the cultivation by each mutant and the respective heterozygous diploid are listed in Table 1. Mutants showed varing levels of acid phosphatase activity in nutrient medium; some of them showed higher activity than strain H-42 (*PHOC*) and some of the diploids produced the enzyme to the same extent as their respective mutants. However, the specific activities of diploids prepared from strain OC-6A, a derivative of strain O27-M66, and strain O49-M22 were significantly lower than those of the original mutants. Similarly, diploids prepared with strains O27-M43 and O38-M58 also showed somewhat lower activities than their respective haploids. A diploid homozygous for the mutant allele of strain O27-M66

TABLE 1. *Acid phosphatase activity of the constitutive mutants and diploids prepared by crossing mutants and the phoC-1 strains*

Strain	Enzyme activity[a]			
	Alone		Diploid[b]	
	Nutrient	Low P_i	Nutrient	Low P_i
H-42 (*PHOC*)	0.058	0.456		
P-28-24C (*phoC-1*)	0.001	0.445		
P-22-14D (*phoC-1*)	0.001	0.500		
O27-M43	0.142	0.487	0.069	0.428
O34-M62	0.008	0.482	0.010	0.581
O34-M65	0.008	0.497	0.009	0.557
O36-M54	0.070	0.581	0.069	0.665
O38-M58	0.110	0.515	0.055	0.593
O49-M21	0.008	0.580	0.009	0.422
O49-M22	0.124	0.533	0.045	0.604
OC-6A[c]	0.076	0.531	0.015	0.515
OC-6A × P-76-4B[c]			0.065	NT[d]
P-32-2B (*phoR*)	0.200	0.461		
P-76-4B × P-32-2B			0.015	NT
P-32-2B × P-28-24C			0.000	0.508

[a] The mutants and the diploids were grown in nutrient and low-P_i media at 30 C for 24 h with shaking. The maximal specific activity produced by each strain during cultivation was expressed as units per unit of optical density of cell suspension at 660 nm.

[b] Diploids were prepared by crossing mutants with *phoC-1*, P-28-24C (α) and P-22-14D (α) unless otherwise noted.

[c] Strains OC-6A and P-76-4B are the haploid derivatives of the α and a mating types, respectively, having the same genotype of the original mutant, O27-M66, for constitutiveness of the acid phosphatase formation.

[d] NT, Not tested.

and a diploid doubly heterozygous for the mutant alleles of strain O27-M66 and *phoR* were also prepared. These diploids were subjected to the enzyme assay described above. The former homozygous diploid showed the same specific activity as strain OC-6A (O27-M66), whereas the latter doubly heterozygous diploid showed a lower specific activity as in the case of the diploid prepared by crossing strain OC-6A with the *phoC-1* strain. Recessiveness of the *phoR* mutation was demonstrated by the assay of the diploid prepared by cross of P-32-2B (*phoR*) to P-28-24C (*phoC-1*) (Table 1). Further derepressed synthesis of acid phosphatase was observed in both haploid and diploid cells grown in low-P_i medium.

Linkage studies. To make further characterization of these dominant, constitutive mutants, each mutant was crossed to the *PHOC* strains. The resulting diploids were sporulated, and four-spored asci were dissected. Each tetrad segregant was tested for acid phosphatase formation on high-P_i medium by staining. Results indicated that these constitutive mutants could be divided into two groups (Table 2). One group frequently segregated *phoC* clones, whereas the other did not. Thus, the dominant nature of the former group, which includes strains O27-M66, O49-M21, and O49-M22, would seem to be attributable either to an operator-constitutive mutation occurring in the regulatory system for the repressible acid phosphatase, or to an extragenic mutation that suppressed *phoC-1*. The second group of constitutive mutations could be attributable to the reversion from *phoC-1* to *PHOC*, or to intragenic suppression in the *phoC* locus. However, a constitutive mutation occurring in the *phoE*-linked operator region, if present, would not be distinguishable from the *phoC-1* to *PHOC* reversion, since the *phoE* locus is closely linked to the *phoC* locus. If the presumed *PHOO* locus exerts a similar function to the operator described in the bacterial system, it would be contiguous to *phoE*, which seems to be the structural gene for the repressible acid phosphatase. However, it was inferred that the presumed *PHOO* mutations occurring in strains O27-M66, O49-M21, and O49-M22 would not be linked to *phoE*, because the mutations occurring in these strains were not linked to the *phoC* locus (Table 2) and *phoC* is linked to *phoE*.

To test the linkage to the other *pho* genes, each of the presumed *PHOO* mutants was crossed with each of the *phoB*, *phoD*, and *phoS* mutants. The diploids were then sporulated,

TABLE 2. *Tetrad segregations in crosses between the dominant constitutive mutants and the PHOC strains*

Cross	Segregation in asci (on high-P_i)		
	++++	+++−	++−−[a]
O27-M43 × H-42[b]	10	0	0
O34-M62 × P-28-24D[b]	17	0	0
O34-M65 × P-28-24D	19	0	0
O36-M54 × P-28-24D	34	0	0
O38-M58 × P-28-24D	14	0	0
O49-M21 × P-28-24D	6	11	6
O49-M22 × P-28-24D	3	5	1
O27-M66 × H-42	3	12	0

[a] The phenotype of each tetrad segregant was tested for its ability to produce acid phosphatase by the staining method.

[b] H-42 and P-28-24D were used as the *PHOC* strains having the a and α mating types, respectively.

and four-spored asci were dissected. Segregants were tested for acid phosphatase formation on high-P_i and low-P_i media by the staining method. The mutant locus in strain O27-M66 is closely linked to the *phoD* locus, since 307 of 311 asci tested showed the parental ditype segregation (2 constitutive; 2 nonproducer) and only 4 tetra-type asci (1 repressible; 1 constitutive; 2 nonproducer) were observed in the cross of OC-6A to O16-M21 (Table 3). However, the other dominant constitutive mutants, O49-M21 and O49-M22, were segregated independently from *phoD*. Although the complete tetrad data are not yet available, owing to poor germination of spores obtained from crosses of the *phoS* mutant by strains O49-M21 and O49-M22, both mutant alleles occurring in O49-M21 and O49-M22 were conjectured to be linked to the *phoS* locus, since no wild-type recombinant appeared from 48 and 49 spores, respectively, randomly isolated from the above crosses.

In all asci from the cross between strains OC-6A (O27-M66) and O16-M6 (*phoS*), more than two segregants per ascus could produce acid phosphatase in low-P_i medium, and there were always two constitutive spores per ascus. On the other hand, in all other crosses that could be analyzed, a 2+/2− segregation pattern was observed for the production of acid phosphatase on low-P_i medium, and less than two segregants per ascus were constitutive. These results clearly support the idea that the double mutant involving any one of these constitutive mutations and either the *phoB* or the *phoD* mutant allele cannot produce the acid phosphatase either in the high-P_i or low-P_i medium. This fact also indicates that these constitutive mutations do not suppress the *phoC-1* allele because, if this were the case, every ascus in the cross between the constitutive mutants and *phoB* or *phoD* would show a 2+/2− segregation in high-P_i medium. All of the results are consistent with the fact that these mutant loci have a specific function in the formation of the repressible acid phosphatase. The site of the dominant, constitutive mutation that occurred in strain O27-M66 was designated as *PHOO*. The segregation pattern in the cross involving the constitutive *PHOO* mutant and the *phoS* mutant can be explained by the fact that the *PHOO* mutation is epistatic over the *phoS* gene. The relationship between *PHOO* and *phoD* was analyzed further.

Role of the phoD gene. In these situations, there are two possibilities for the function of the *phoD* gene. First, the product from the *phoD* gene could be a part of the repressible acid phosphatase molecule and also probably a part of the repressible alkaline phosphatase. Alternatively, the *phoD* gene might exert a positive control in the formation of these repressible phosphatases. To distinguish these possibilities,

TABLE 3. *Tetrad analysis for testing the linkage between the site of the dominant constitutive mutation and the phoB, phoD, and phoS loci*[a]

Cross[b]	Segregation in asci				
	++++[c] ++−−[d]	+++− ++−−	++−− ++−−	++−− +−−−	++−−[e] −−−−
OC-6A[f] × O16-M3 (*phoB*)	0	0	1	12	0
OC-6A × O16-M21 (*phoD*)[g]	0	0	307	4	0
OC-6A × O16-M6 (*phoS*)	2	12	2	0	0
O49-M21 × O16-M3 (*phoB*)	0	0	1	9	2
O49-M21 × O16-M21 (*phoD*)	0	0	1	15	2
O49-M21 × O16-M6 (*phoS*)	Poor germination[h]				
O49-M22 × O16-M3 (*phoB*)	0	0	3	11	2
O49-M22 × O16-M21 (*phoD*)	0	0	3	19	3
O49-M22 × O16-M6 (*phoS*)	Poor germination[h]				

[a] Tetrad analyses for testing the linkage between the sites of mutation and *phoE* were not performed because those mutations were not linked with *phoC* (cf. Table 2), and *phoC* is closely linked with *phoE*.

[b] All the standard strains have the *phoC-1* allele.

[c] On low P_i.

[d] On high P_i.

[e] Each segregant was tested for its ability to produce acid phosphatase by the staining method.

[f] Strain OC-6A is the haploid clone having the same genotype as strain O27-M66.

[g] Cross no. P-82; cf. text and Tables 4, 5, and 6 for further analyses.

[h] Complete tetrad data are not available yet due to the poor germination of spores in these hybrids. However, both the mutant alleles occurring in strains O49-M21 and O49-M22 were found to be linked to the *phoS* locus by random spore analysis.

we isolated the temperature-sensitive *phoD* mutant O50-M48 from the *phoC-1* strain by EMS treatment. That the mutant locus in strain O50-M48 is an allele of the *phoD* locus was confirmed by the fact that no complementation was shown between strain O50-M48 and the standard *phoD* mutant, but complementation was shown with the other *pho* mutations lacking the enzyme activity. Furthermore, no recombination occurred between the mutant allele in strain O50-M48 and the *PHOO* mutation for the few asci tested. The temperature-sensitive *phoD* mutant O50-M48 and the wild-type (*PHOD*) strain P-28-24C were grown in low-P_i medium at 25 and 35 C with shaking. The time course of the appearance of the acid phosphatase activity was followed during the cultivation. Both strains O50-M48 and *PHOD* showed virtually the same growth pattern in low-P_i medium (Fig. 1). However, the time courses of the acid phosphatase formation were quite different for these strains in that the wild type could produce the acid phosphatase at both temperatures whereas the mutant strain could produce some of the enzyme activity at 25 C but none at 35 C.

The thermolability of the acid phosphatases produced by strains O50-M48 and *PHOD* was examined with a cell extract as the enzyme. Each strain was grown in low-P_i medium, at 25 C for strain O50-M48 and at 30 C for the wild-type strain, for 20 h with shaking. A cell extract prepared from each strain was preincubated at 55 C for various periods and then rapidly cooled in an ice bath. The remaining acid phosphatase activity was determined by using *p*-nitrophenylphosphate as substrate. The results (Fig. 2) indicate no significant difference in the heat inactivation of the acid phosphatases between two samples prepared from strain O50-M48 and the *PHOD* strain (P-28-24C). A kinetic study was also performed with the same substrate, and the same K_m value was obtained in the Lineweaver-Burk plots for both enzyme preparations (Fig. 3). Extracts were also prepared from the cells of four independent revertants from strain P-82-53C (*phoO phoD*; see Tables 4, 5, and 6) and were analyzed as described above. No significant differences were observed in the thermolability and K_m value of the acid phosphatase activity produced by these revertants and the wild-type strain (Fig. 2, 3). All of these results suggest that the *phoD* locus is not the structural gene for the repressible acid phosphatase, but is a regulatory gene.

cis dominance of the PHOO mutation. Assuming that the *PHOO* locus is the operator for the *phoD* locus, it should control the expression of the *phoD* gene only in the *cis* position. To test this possibility, it is necessary to obtain a *PHOO phoD* double mutant. Such a strain should be obtainable in the tetra-type ascus from a cross of the *PHOO* and *phoD* mutants (OC-6A × O16-M21; cross no. P-82) (Table 3). Among the tetra-type asci in this cross, a set of tetrad clones from the P-82-53 ascus was selected for further investigation. Judging from the phenotypes shown in Table 4, the genotypes

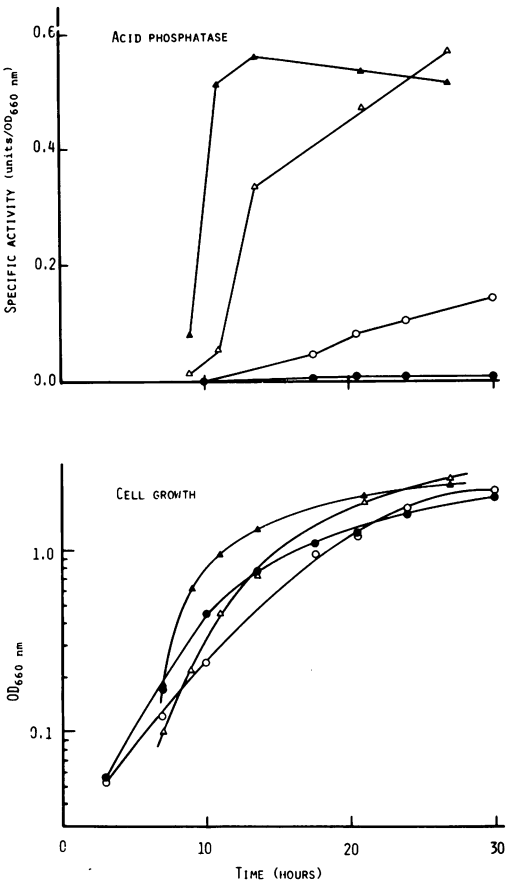

FIG. 1. *Time courses of the formation of acid phosphatase in strain PHOD and the temperature-sensitive phoD mutant. The PHOD strain P-28-24C and the temperature-sensitive phoD mutant O50-M48 were grown in low-P_i medium at 25 and 35 C with shaking. At the indicated time, a sample of the culture was taken for determination of cell density and acid phosphatase activity. The enzyme activity was assayed by using a suspension of intact cells as the enzyme source. Specific activity was expressed as units per optical density unit at 660 nm. Symbols:* O, *O50-M48 at 25 C;* ●, *O50-M48 at 35 C,* Δ, *P-28-24C at 25 C; and* ▲, *P-28-24C at 35 C.*

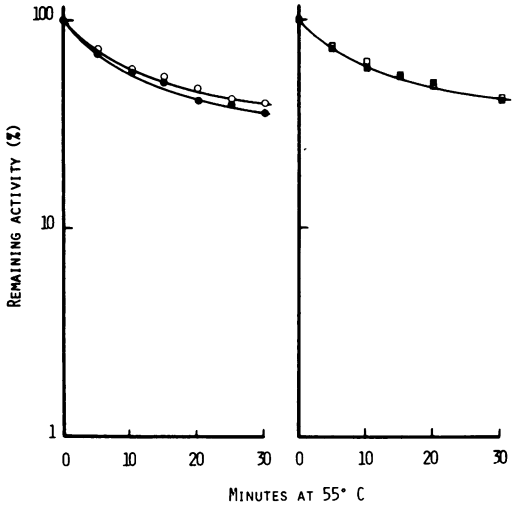

FIG. 2. *Heat inactivation of the acid phosphatase activity in cell extracts prepared from the PHOD strains and the temperature-sensitive phoD mutant. Crude extract was prepared from the cells of each strain grown, in low-P_i medium for 20 h, to early stationary phase at 25 C for the temperature-sensitive phoD mutant O50-M48 and at 30 C for the wild-type strain. Extracts were also prepared from cells of the revertants from the phoD mutant, i.e., P-82-53CR49 and P-82-53CR50 (cf. Tables 5 and 6), cultivated at 30 C for early stationary phase. Each extract was diluted with 0.01 M acetate buffer (pH 4.0) to give 0.1 to 0.5 U of enzyme activity per ml, and a portion (0.5 ml) of the diluted extract was preincubated at 55 C for the indicated period and was rapidly cooled in an ice bath. Enzyme activity without preincubation was scored as 100%, and the experimental values were expressed as percentage of this value. Symbols:* ○, *O50-M48;* ●, *P-28-24C (wild, phoC-1);* □, *P-82-53CR49 (phoD revertant); and* ■, *P-82-53CR50 (phoD revertant).*

of strains P-82-53A and P-82-53D were *PHOO PHOD* and *phoO PHOD*, respectively. Thus, the genotypes of P-82-53B and P-82-53C should be *PHOO phoD* and *phoO phoD*, respectively. Although these two segregants do not show acid phosphatase activity either on low-P_i or high-P_i medium, it is possible to distinguish them genetically. When *PHOO phoD* and *phoO phoD* are crossed with the wild-type *phoO PHOD* strain, a constitutive clone may be segregated only from the former cross. This was tested by crossing strains P-82-53B and P-82-53C to the wild-type strain, P-28-24C. Of the 403 asci from the P-82-53B × P-28-24C cross, 4 contained a constitutive clone. On the other hand, no constitutive clone was obtained in the cross involving strains P-82-53C and P-28-24C in the 432 asci so far examined. Hence, the genotypes of strains P-82-53B and P-82-53C were determined to be *PHOO phoD* and *phoO phoD*, respectively.

This was confirmed further by isolating the revertants from strains P-82-53B and P-82-53C. Since P-82-53B showed poor growth in minimal medium, three segregants, P-84-392B, P-84-393B, and P-84-394A, were selected from the cross between strains P-82-53B and P-28-24C. These three clones should have the same genotype as strain P-82-53B, because the tetrads from which they were selected showed a 2+/2− segregation on low-P_i medium and 0+/4− on high-P_i medium. Each of these strains was grown in nutrient medium at 30 C for 24 h. The cells were washed with sterilized water and resuspended in the original volume of sterilized water. A 0.1-ml volume of the resultant cell suspension was spread on the plate containing low-P_i medium, and incubated at 35 C for several days. Spontaneous revertants that produced the acid phosphatase on low-P_i medium were inspected by the staining method, and were isolated and purified by repeated streaking. Seven revertants from the P-82-53B derivatives and four revertants from strain P-82-53C were isolated. That the site of mutation in these revertants is the *phoD* locus was confirmed by genetic analysis of hybrids ob-

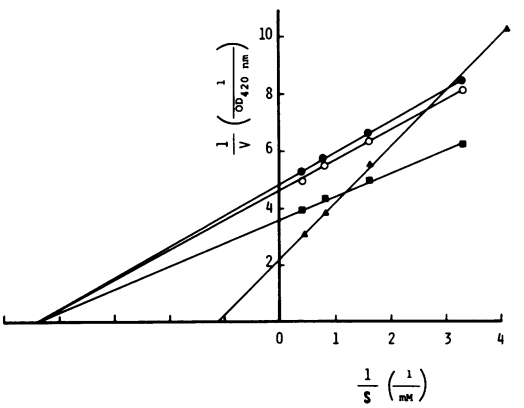

FIG. 3. *Lineweaver-Burk plots of the acid phosphatase prepared from cells of the PHOD strain (P-28-24C), the temperature-sensitive phoD mutant (O50-M48), and the revertant from phoD mutant (P-82-53CR50), with p-nitrophenylphosphate as substrate. Extract was prepared from cells grown in low-P_i medium for 20 h at 25 C for O50-M48 or at 30 C for the other strains, with shaking. Each extract was dialyzed against 0.01 M acetate buffer (pH 4.0) and diluted to give about 0.1 U of enzyme activity per ml with the same buffer. Symbols:* ○, *O50-M48;* ●, *P-28-24C;* ■, *P-82-53CR50; and* ▲, *the constitutive enzyme from the PHOC strain (H-42).*

TABLE 4. *Determination of the genotype of each tetrad clone in one of the tetratype asci from the diploid, P-82, obtained by crossing between the phoD and PHOO mutants*

Strain	Mating type	Enzyme formation[a]		Segregation of a constitutive clone from the hybrid	Expected genotype
		High P_i	Low P_i		
P-82-53A	*a*	+	+	Not tested	*PHOO PHOD*
P-82-53B	*α*	−	−	4/403[b]	*PHOO phoD*
P-82-53C	*α*	−	−	0/432	*phoO phoD*
P-82-53D	*a*	−	+	Not tested	*phoO PHOD*

[a] Ability to produce acid phosphatase activity was tested by the staining method.
[b] Number of asci containing a constitutive clone among tetrad segregants per total asci tested in each cross with P-28-24C (*phoO PHOD*).

tained by crossing these revertants with the *phoC-1 phoO PHOD* strain. Since no segregant showing the same phenotype as the *phoD* mutant, i.e., lacking acid phosphatase activity on low-P_i medium, was obtained in these crosses (Table 5), it was concluded that these reversions occurred in the *phoD* locus. These revertants were grown in nutrient and low-P_i media at 30 C with shaking, and each maximal specific activity attained during the cultivation were determined (Table 6). The data showed clearly that all of the revertants from the P-82-53B derivatives are constitutive and none of the revertants from strain P-82-53C is constitutive. These results are also consistent with the idea that the genotypes of strains P-82-53B and P-82-53C are *PHOO phoD* and *phoO phoD*, respectively.

Diploids having the *PHOO PHOD/phoO PHOD* and *PHOO phoD/phoO PHOD* genotypes were prepared. These strains were grown in nutrient and low-P_i media at 30 C with shaking. The acid phosphatase activity was assayed by using the intact cell suspension as an enzyme source. Maximal specific activities obtained during culturing were determined (Table 7). The diploid *PHOO phoD/phoO PHOD* produced the acid phosphatase in low-P_i medium but not in nutrient medium. On the other hand, the diploid *PHOO PHOD/phoO PHOD* produced the acid phosphatase both in nutrient and low-P_i media. Thus, it was concluded that *PHOO* controls *PHOD* only in the *cis* position.

TABLE 5. *Tetrad analyses of the diploids obtained by crossing the revertants from the P-82-53B derivatives and P-82-53C with the phoC-1 phoO PHOD strains*

Cross	Total asci tested	Phenotypic segregation in asci[a] on low-P_i medium (+:−)				
		4:0	3:1	2:2	1:3	0:4
P-84-392B[b]R1 × P-28-24B[c]	33	33	0	0	0	0
P-84-392BR2 × P-28-24B	20	20	0	0	0	0
P-84-392BR3 × P-28-24B	20	20	0	0	0	0
P-84-393B[b]R1 × P-28-24B	18	18	0	0	0	0
P-84-394A[b]R1 × P-28-24B	18	18	0	0	0	0
P-82-53CR49 × P-28-24C[c]	23	23	0	0	0	0
P-82-53CR50 × P-28-24C	41	41	0	0	0	0
P-82-53CR51 × P-28-24C	33	33	0	0	0	0
P-82-53CR52 × P-28-24C	25	25	0	0	0	0

[a] Phenotype determined by the staining method.
[b] Since P-82-53B showed poor growth in low-P_i medium, the segregants P-84-392B, P-84-393B, and P-84-394A were selected from the cross between strains P-82-53B and P-28-24C (*phoO PHOD;* cf. Table 4) and have the same genotype as P-82-53B with respect to acid phosphatase formation.
[c] P-28-24B and P-28-24C were used as the *phoC-1 phoO PHOD* strains having the *α* and *a* mating types, respectively.

DISCUSSION

Positive control has been well defined in bacterial systems, especially for arabinose metabolism in *E. coli* (7), and in a viral system (12). This mode of control could be ascribed to a certain protein factor(s) that interacts with ribonucleic acid polymerase (1, 14), or with a specific stretch of deoxyribonucleic acid around the initiation site of the gene (8). In eukaryotic systems, some investigations have suggested the existence of a positive control (11, 17), although the mode of action of the controlling factors remains unknown. Marzluf and Metzenberg (16) have suggested that the *cys-3* locus, involved in the regulation of sulfur metabolism in *Neurospora crassa*, exerts positive control, since the *cys-3* mutation results in a pleiotropic-nega-

TABLE 6. *Acid phosphatase formation by the revertants from the P-82-53B derivatives and strain P-82-53C*

Strain	Expected[a] genotype	Enzyme activity[b]	
		Nutrient	Low P_i
P-82-53B	*PHOO phoD*	0.000	0.000
P-84-392B[c]	*PHOO phoD*	0.000	0.000
P-84-392BR1		0.081	0.750
P-84-392BR2		0.064	0.685
P-84-392BR3		0.079	0.730
P-84-393B[c]	*PHOO phoD*	0.000	0.000
P-84-393BR1		0.101	0.800
P-84-393BR2		0.097	0.730
P-84-393BR3		0.096	0.720
P-84-394A[c]	*PHOO phoD*	0.000	0.000
P-84-394AR1		0.079	0.745
P-82-53C	*phoO phoD*	0.000	0.000
P-82-53CR49		0.000	0.319
P-82-53CR50		0.000	0.324
P-82-53CR51		0.000	0.345
P-82-53CR52		0.000	0.324

[a] Expected genotype of each original strain was deduced from the phenotype of the respective revertants.

[b] Each strain was grown in nutrient and low-P_i media at 30 C with shaking. Acid phosphatase activities were assayed at appropriate intervals during the cultivation by using a suspension of intact cells as the enzyme source. The maximal specific activity (units per optical density unit at 660 nm) attained during the cultivation is shown.

[c] The original clones for isolation of revertants were the derivatives from strain P-82-53B (see footnotes to Table 5).

TABLE 7. *cis dominancy of the PHOO-PHOD complex*

Experiment[a]	Genotype of diploid	Enzyme activity[b]	
		Nutrient	Low P_i
1	*phoO PHOD/phoO PHOD* (wild)	0.000	0.505
2	*PHOO PHOD/phoO PHOD*	0.012	0.515
3	*PHOO PHOD/phoO PHOD*	0.05	0.515
4	*PHOO phoD/phoO PHOD*	0.000	0.245
5	*PHOO phoD/phoO PHOD*	0.000	0.290
6	*PHOO phoD/phoO PHOD*	0.000	0.335
7	*PHOO phoD/phoO PHOD*	0.000	0.280

[a] Each experiment was run with the independent diploid strain.

[b] Diploid strains were grown in nutrient and low-P_i media at 30 C with shaking. Acid phosphatase activities were assayed at appropriate intervals during the cultivation by using a suspension of intact cells as the enzyme source. The maximal specific activity (units per optical density unit at 660 nm) attained during the cultivation is shown.

tive phenotype in three structural genes coding for sulfate permease, aryl sulfatase, and choline sulfatase. Furthermore, a temperature-sensitive *CYS-3* revertant occurring in the *cys-3* locus produces the normal enzymes. The *leu-3* locus of leucine biosynthesis (21), the *qa-1* locus of quinic acid catabolism (27) in *N. crassa*, and the *amdR* locus of acetamidase system in *Aspergillus nidulans* (5) can exist in two mutant states, negative and constitutive, which may correspond to *araC*$^-$ and *araC*^{c} in the arabinose system of *E. coli* (7). The mutation in these regulatory genes, in addition to the fact that mutations in them do not seem to affect the structure of the enzyme molecule, suggested that these genes exert positive control over the respective system.

The *phoD* locus in the acid phosphatase system in *S. cerevisiae* is also thought to have a positive function in enzyme synthesis for the following reasons. (i) The mutation occurring in the *phoD* locus gave rise to simultaneous loss in capacity to derepress both acid and alkaline phosphatases (26). (ii) Acid phosphatase activities of cell extracts prepared from cells of the temperature-sensitive *phoD* mutant cultivated at permissive temperature (25 C), or cells of the *PHOD* revertants, showed the same thermolability and K_m value for *p*-nitrophenylphosphate as the normal repressible enzyme described in this paper.

A mutation resulting in the constitutive phenotype was found to be in a gene closely linked to the *phoD* locus designated *PHOO*. Since the *PHOO* mutation was dominant, although only partially, over the wild-type allele and showed *cis* dominance over the *PHOD* gene, it was suggested that the *PHOO* locus worked as an operator for the *phoD* locus. Other than the *C* gene in the galactose-utilizing system (4) and *cpa10* in the carbamoylphosphate synthetase of the agrinine biosynthetic pathway of *S. cerevisiae* (25), there is very limited evidence indicating the existence of operator loci in eukaryotic system. The recombination frequency between a pair of mutant alleles of *PHOO* and *phoD* was 8/714 (number of asci occurring recombination per total number of asci tested; Tables 3 and 4). This value is roughly comparable to that between *C* and *gal4* (1/82 [4]) and between *cpa10* and *cpa1* (6/242 [25]).

Partial dominance observed in the *PHOO* mutant (Table 1) might not be caused by the difference of the cell concentration at the same optical density, at 660 nm, between the haploid and diploid strains, because the diploid homozygous for *PHOO* gave almost the same specific

activity as the haploid mutant. Partial dominance of the constitutive allele was found in the quinic acid catabolic system of *N. crassa* described by Valone et al. (27). They stated that the *qa-1* gene exerted positive control and that the dominant constitutive mutant, *qa-1*^{c}, could be isolated directly from a *qa-1* mutant, which could not induce three enzymes (catabolic dehydroquinase, quinate dehydrogenase, and 5-dehydroshikimate dehydrase) from their respective structural genes. Since the *qa-1* gene was effective both in *trans* and *cis* position in heterokaryon, and because of the existence of intermediate activities of enzymes in certain heterokaryons involving *ga-1*^{c} and *qa-1*$^+$ nuclei, they suggested that the regulatory product of the *qa-1* gene is a multimeric protein capable of producing hybrids with homologous mutant protein. The absence of the clear dominance-recessiveness relationship in multimeric enzyme formation was described for the threonine dehydratase system of *S. cerevisiae* by Zimmermann et al. (29). The reduced acid phosphatase activity shown by the *PHOO phoD/phoO PHOD* diploid in low-P_i medium (Table 7) might be explained as a similar subunit interaction or as a dosage effect of the *phoD* gene. However, partial dominance in the case of the *PHOO* mutation can not be explained by the same mechanism since *PHOO* is suspected to work as an operator for the *phoD* gene.

That the *phoR phoD* double mutant could not produce the repressible acid phosphatase, and the presence of the *PHOO* locus contiguous to *phoD* suggest that the *phoR* gene controls the formation of the enzyme via the expression of the *phoD* gene whose recognition site for the *phoR* product is the *PHOO* locus. The epistatic nature of the *PHOO* mutation to the *phoS* gene suggested by the genetic data listed in Table 3 is also consistent with the above argument, since we had previously proposed (26) that the *phoS* gene controls the expression of the *phoR* gene. Another regulatory system in *S. cervisiae*, the *gal* system, which was extensively studied by Douglas and Hawthorne (4), contains three regulatory genes, *i*, *C*, and *gal4*. They suggested that the *C* and *gal4* consist of an operon and that the *i* specifies the repressor whose site of action is *C*. The *C* gene recognizes the repressor and controls the expression of the contiguous gene, *gal4*, which in turn controls the expression of the structural genes, *gal1*, *gal7*, and *gal10*, which code the three enzymes in the galactose pathway. Comparing the genetic regulatory mechanisms working in the repressible acid phosphatase and the galactose pathway enzymes, one finds that those regulatory systems are controlled in a similar manner, at least in part. It seems likely that the functions of the *phoR*, *PHOO*, and *phoD* genes correspond to those of *i*, *C*, and *gal4*, respectively. Although it can not be generalized immediately, these results strongly suggest that a gene cluster, such as *phoO-PHOD* or *c-GAL4*, that mediates the information between the regulator gene(s) and the structural gene(s) is operating in the regulatory system of *S. cerevisiae*.

Dominant constitutive mutants other than the *PHOC* revertants and the *PHOO* mutation were also isolated. Preliminary results indicated that their mutation sites are linked to the *phoS* locus. Further study with these mutants is now underway.

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