REGULATION OF REPRESSIBLE ACID PHOSPHATASE BY CYCLIC AMP IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

One of the *cyr1* mutants (*cyr1-2*) in yeast produced low levels of adenylate cyclase and cyclic AMP at 25° and was unable to derepress acid phosphatase. Addition of cyclic AMP to the *cyr1-2* cultures elevated the level of repressible acid phosphatase activity. The *bcy1* mutation, which suppresses the *cyr1-2* mutation by allowing activity of a cyclic AMP-independent protein kinase, also allows acid phosphatase synthesis without restoring adenylate cyclase activity. The *CYR3* mutant had structurally altered cyclic AMP-dependent protein kinase and was unable to derepress acid phosphatase. The *cyr1* locus was different from *pho2*, *pho4* and *pho81*, which were known to regulate acid phosphatase synthesis. Mutants carrying *cyr1-2* and *pho80*, *PHO81^c*, *PHO82* or *pho85* mutations, which confer constitutive synthesis of repressible acid phosphatase, produced acid phosphatase. The *cyr1-2* mutant produced significantly low levels of invertase and α-D-glucosidase. These results indicated that cyclic AMP-dependent protein kinase exerts its function in the synthesis of repressible acid phosphatase and other enzymes.

CYCLIC AMP (cAMP) has been shown to be involved in the control of a variety of metabolic processes in eukaryotes as well as prokaryotes (ROBISON, BUTCHER and SUTHERLAND 1971). Evidence to show cAMP action in *Saccharomyces cerevisiae* has been obtained by isolating mutants altered in cAMP metabolism. Yeast mutants deficient in adenylate cyclase activity (*cyr1*) require cAMP for growth (MATSUMOTO *et al.* 1982a). Other mutants altered in the regulatory subunit of cAMP-dependent protein kinase (*CYR3*) also require cAMP (UNO, MATSUMOTO and ISHIKAWA 1982). Our studies on these mutants indicated that cAMP is an essential factor for yeast cells to proceed through the cell cycle via the activation of cAMP-dependent protein kinase (MATSUMOTO *et al.* 1982a; MATSUMOTO, UNO and ISHIKAWA 1983). In addition, it is known that cAMP is involved in controlling the expression of genes regulated at the level of transcription in prokaryotes and eukaryotes (PASTAN and ADHYA 1976; WILLIAMS, TSANG and MAHBUBANI 1980; CHRAPKIEWICZ, BEALE and GRANNER 1982). It has been shown that cAMP may not be involved in catabolite repression but has a stimulative effect on constitutive synthesis of some enzymes in yeast (MATSUMOTO *et al.* 1982b, 1983). However, the regulatory role of cAMP on gene expression in yeast is not yet clear.

The repressible acid phosphatase system in *S. cerevisiae* offers a unique opportunity to investigate the effect of cAMP in gene expression. *S. cerevisiae* has two species of acid phosphatase (EC 3.1.3.2); one is a constitutive enzyme coded by the *PHO3* gene, and the other is a repressible enzyme coded by the *PHO5* gene (TOH-E *et al.* 1973; TOH-E, KAKIMOTO and OSHIMA 1975). Synthesis of repressible acid phosphatase is negatively controlled by P_i levels present in the growth medium (TOH-E *et al.* 1973). The repression of enzyme synthesis has been shown by TOH-E, INOUYE and OSHIMA (1982) to be regulated by the *PHO2*, *PHO4*, *PHO80*, *PHO81* and *PHO85* genes. Strong support for transcriptional regulation of the acid phosphatase gene has been provided by recent demonstrations that synthesis of mRNA for repressible acid phosphatase polypeptides was controlled by P_i and that several yeast genes under P_i control were isolated (BOSTIAN *et al.* 1980; KRAMER and ANDERSEN 1980; ROGERS, LEMIRE and BOSTIAN 1982).

In this paper, we report that one of the cAMP-requiring mutants (*cyr1-2*) lacks production of the repressible acid phosphatase under conditions that permit slow growth and describe the involvement of cAMP-dependent protein kinase in the synthesis of repressible acid phosphatase.

MATERIALS AND METHODS

Yeast strains: The *S. cerevisiae* strains used in this study were derived from the *pho3-1* strain (P-28-24C) (KANEKO, TOH-E and OSHIMA 1982) and are listed in Table 1. All strains except for 038-M58 and AM163-1A carry the *pho3-1* mutation to eliminate the constitutive acid phosphatase activity (TOH-E *et al.* 1973; TOH-E, KAKIMOTO and OSHIMA 1975).

Media: General usage and composition of minimal, YPGlu, YPGly, YPGlyMal and sporulation media were described previously (NOGI *et al.* 1977; MATSUMOTO, TOH-E and OSHIMA 1981). Low-P_i medium was prepared by the method of RUBIN (1974) with minor modification; 10 g of yeast extract (Difco laboratories) and 20 g of peptone (Difco Laboratories) were dissolved in deionized water to a volume of 1 liter, and to this was added 10 ml of 1 M $MgSO_4$ and then 10 ml of concentrated ammonium hydroxide. The solution was mixed well and left to stand at room temperature for 30 min. The precipitate formed was removed by filtration with Whatman no. 1 filter paper. The filtrate was supplemented with 20 g of glucose after adjustment to pH 5.8 by dropwise addition of concentrated HCl, and the whole solution was autoclaved for sterilization. High-P_i medium was low-P_i medium plus 2 g of KH_2PO_4 per liter. Low-P_i·cAMP and high-P_i·cAMP media were low-P_i and high-P_i media supplemented with filter-sterilized cAMP (1 mM), respectively. Minimal low-P_i medium was the same as Burkholder's synthetic medium in which L-asparagine alone is used as nitrogen source and 1.5 g of KH_2PO_4 was replaced by 30 mg of KH_2PO_4 and 1.5 g of KCl (TOH-E *et al.* 1973).

Genetic techniques: The methods for genetic analyses were described by NOGI *et al.* (1977).

Detection of phosphatase activity of colonies: Acid phosphatase activity of colonies grown on agar media was detected by a staining method based on the diazo-coupling reaction (TOH-E and OSHIMA 1974).

Preparation of cell-free extract: All experiments were carried out at 4°. Cells grown at 25° for 24 hr with shaking were harvested by centrifugation and suspended in buffer T containing 50 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA, 1 mM mercaptoethanol and 0.5 mM phenylmethylsulfonylfluoride. The suspension obtained was homogenized with an Aminco French pressure cell press (J5-598A) at 10,000 psi. The resulting homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant fluid was used as a crude extract.

Enzyme assays: Adenylate cyclase was assayed as described by UNO, NYUNOYA and ISHIKAWA (1981). One unit of the enzyme activity was defined as the amount that produced 1 pmol of cAMP in 1 min at 30°. The activity of acid phosphatase was assayed using intact cells as the enzyme

TABLE 1

List of yeast strains used

Strain	Genotype	Source (reference)
AL224-4A	**a** *pho3-1 leu1*	KANEKO, TOH-E and OSHIMA (1982)
F10D	*α pho3-1*	MATSUMOTO, TOH-E and OSHIMA (1978)
G435-5B	*α pho3-1 leu1*	MATSUMOTO, UNO and ISHIKAWA (1983)
KYC95	**a** *pho3-1 PHO83 trp5*	Obtained from Y. KANEKO
KYC146	*α pho3-1 pho85 arg6 leu1*	Obtained from Y. KANEKO
KYC168	*α pho3-1 pho80-3 his6*	Obtained from Y. KANEKO
KYC188	*α pho3-1 PHO81[c] his2 cdc14*	Obtained from Y. KANEKO
038-M58	**a** *PHO3+*	Obtained from A. TOH-E
P178-6C	**a** *pho3-1 PHO82-7*	TOH-E, INOUYE and OSHIMA (1981)
AM9-8B	**a** *bcy1 pho3-1*	MATSUMOTO *et al.* (1982)
AM26-2A	**a** *cyr1-2 pho3-1*	MATSUMOTO, UNO and ISHIKAWA (1983)
AM33-1A	**a** *CYR3-1 pho3-1 leu1*	UNO, MATSUMOTO and ISHIKAWA (1982)
AM69-7D	*α bcy1 pho3-1 leu1*	MATSUMOTO, UNO and ISHIKAWA (1983)
AM70-1D	*α cyr1-2 pho3-1 leu1*	MATSUMOTO, UNO and ISHIKAWA (1983)
AM81-1C	**a** *cyr1-2 bcy1 pho3-1*	Segregant from AM26-2A × AM69-7D
AM104-1C	*α cyr1-2 pho3-1 leu1*	Segregant from AM26-2A × G435-5B
AM110-4C	*α cyr1-2 pho3-1 leu1*	Segregant from AM70-1D × AL224-4A
AM110-4D	**a** *cyr1-2 pho3-1 leu1*	Haploid clone from the same family of AM110-4C
AM139-5B	*α cyr1-2 pho3-1 pho80-3*	Segregant from KYC168 × AM110-4D
AM140-1C	*α cyr1-2 pho3-1 PHO81[c]*	Segregant from KYC188 × AM110-4D
AM141-1A	*α cyr1-2 pho3-1 PHO82-7*	Segregant fromP178-6C × AM110-4C
AM142-5C	**a** *cyr1-2 pho3-1 PHO83*	Segregant from KYC95 × AM110-4C
AM143-1C	*α cyr1-2 pho3-1 pho85 leu1*	Segregant from KYC146 × AM110-4D
AM163-1A	**a** *cyr1-2 PHO3+*	Segregant from 038-M58 × AM110-4C

source and *p*-nitrophenylphosphate as the substrate, as described by TOH-E *et al.* (1973). Cells cultivated in YPGlu medium by shaking at 25° for 24 hr were harvested, washed and suspended in the same volume of sterilized water. A portion of the cell suspension was inoculated into the indicated medium to the initial optical density at 660 nm (OD_{660}) of about 0.05 and shaken at 25°. Measurements of acid phosphatase activity were made on cultures with an OD_{660} of 1.5–2.0. One unit of acid phosphatase was defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per min under the assay conditions. Invertase and α-D-glucosidase activities were determined and expressed as described previously (MATSUMOTO, TOH-E and OSHIMA 1981).

cAMP assay: cAMP content was measured by using a cAMP assay kit (Amersham International) as described by UNO, NYUNOYA and ISHIKAWA (1981).

Photoaffinity labeling: The amount of cAMP-binding protein was measured by the 8-azidoadenosine 3′:5′-[^{32}P]monophosphate (8-N_3-[^{32}P]cAMP) (ICN) photoaffinity labeling method described by WALTER *et al.* (1977).

Protein measurement: Protein was measured by the method of LOWRY *et al.* (1951) using bovine serum albumin as the standard.

RESULTS

Acid phosphatase levels in cyr1-2 *mutants*: MIZUNAGA (1980) demonstrated previously that cAMP promoted acid phosphatase synthesis in yeast and was unable to overcome repression of the enzyme synthesis by inorganic phosphate. We have isolated a temperature-sensitive cAMP-requiring mutant, *cyr1-2*, in yeast and investigated the effect of cAMP on acid phosphatase synthesis. This

TABLE 2

Acid phosphatase activity, adenylate cyclase activity and cAMP level of wild-type and mutant strains

Strain	Relevant genotype	Acid phosphatase (units/$OD_{660\ nm}$)				YPGlu	
		Low P_i	Low-P_i·cAMP	High-P_i	High-P_i·cAMP	Adenylate cyclase (units/mg protein)	cAMP level (pmol/mg protein)
038-M58	+ *PHO3*+	0.11	0.14	0.02	0.02	6.7	1.9
AM163-1A	*cyr1-2 PHO3*+	0.01	0.02	0.01	0.02	0.2	0.2
F10D	+ *pho3-1*	0.14	0.17	<0.01	<0.01	6.6	2.0
AM110-4D	*cyr1-2 pho3-1*	<0.01	0.06	<0.01	<0.01	0.3	0.4
AM9-8B	*bcy1 pho3-1*	0.13	0.13	<0.01	<0.01	6.9	1.9
AM33-1A	*CYR3-1 pho3-1*	<0.01	0.02	<0.01	<0.01	7.8	2.1

mutant is able to grow on YPGlu medium at 25° but requires cAMP for growth at 35°. The *cyr1-2* mutant cells grown at 25° produced significantly low levels of adenylate cyclase activity and cAMP (Table 2), and those grown at 35° produced no detectable adenylate cyclase and cAMP. The *cyr1* mutants carry lesions in the structural gene for adenylate cyclase (MATSUMOTO, UNO and ISHIKAWA 1984). The mutant strain AM163-1A (*cyr1-2 PHO3*+) possessed the same level of constitutive acid phosphatase activity as the wild-type strain 038-M58 (*CYR1*+ *PHO3*+) under repressed conditions at 25° (Table 2). Strain 038-M58 was derepressed for acid phosphatase activity, whereas AM163-1A was not. Strain F10D (*CYR1*+ *pho3-1*) and AM110-4D (*cyr1-2 pho3-1*) had no constitutive acid phosphatase under repressed conditions at 25° (Table 2). Strain F10D was derepressed for acid phosphatase, but AM110-4D produced no detectable level of acid phosphatase activity even under derepressing conditions at 25° (Table 2). These results suggest that the *cyr1-2* mutant was apparently unable to derepress acid phosphatase. Addition of cAMP to the growth media elevated the levels of repressible acid phosphatase activity in the wild-type and *cyr1-2* strains (Table 2).

Diploid strain (AM148) constructed by a cross of AM110-4D (**a** *cyr1-2 pho3-1*) with F10D (α *CYR1*+ *pho3-1*) was sporulated, and four-spored asci were dissected. Tetrad analysis of the diploid showed a 4+:0− segregation for growth on YPGlu medium at 25° in the 18 asci tested, whereas they showed a 2+:2− segregation on the same medium at 35°. Six of 18 tetrads were randomly selected, and the Pho phenotype and growth characteristics of segregants were determined as shown in Figure 1. Segregation of the Pho phenotype in the 18 asci tested was 2+:2− on low-P_i and minimal low-P_i media at 25°, and they gave only parental ditype with regard to the Pho^- phenotype and growth on YPGlu medium at 35°. These results indicate that the *cyr1-2* mutation affects synthesis of repressible acid phosphatase.

To confirm that the *cyr1-2* mutant cannot derepress acid phosphatase, the time course of acid phosphatase appearance in cultures of the wild-type

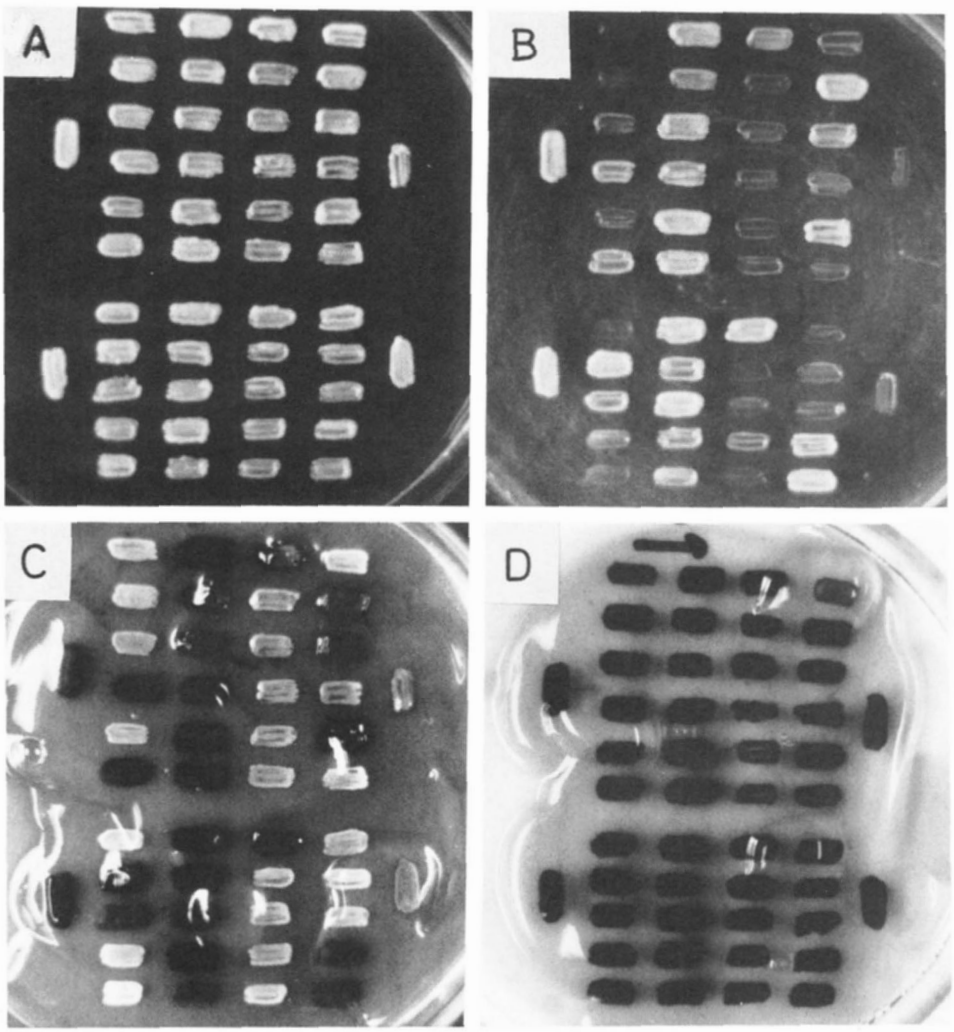

FIGURE 1.—Segregation in tetrads derived from *cyr1-2*/+ and *CYR3-1*/+ diploids. Streaks of the mutant and the wild-type cells are on the left and right, respectively. Horizontal groups of four are members of one tetrad. Six tetrads shown in the upper part of each plate were derived from diploids constructed by the AM110-4D (**a** *cyr1-2 pho3-1*) × F10D (*α CYR3+ pho3-1*) cross, and five tetrads shown in the lower part were derived from diploids constructed by the AM33-1A (**a** *CYR3-1 pho3-1*) × G435-5B (*α CYR3+ pho3-1*) cross. All plates were incubated for 24 hr. A, Cell growth on YPGlu medium at 25°. B, Cell growth on YPGlu medium at 35°. C, Acid phosphatase activity tested by the staining method on low-P_i medium at 25°. D, Acid phosphatase activity tested by the staining method on low-P_i·cAMP medium at 25°. Mutants streaks are white, and wild-type streaks are red on low-P_i medium.

(AM148-1C) and *cyr1-2* (AM148-1D) segregants grown in low-P_i and low-P_i·cAMP media was examined (Figure 2). The *cyr1-2* cells grew more slowly than the wild-type cells in low-P_i medium at 25°; the generation time of the *cyr1-2* cells was prolonged to 4 hr, in comparison with that of the wild-type cells of 2.5–3 hr. The *cyr1-2* cells were unable to derepress acid phosphatase even

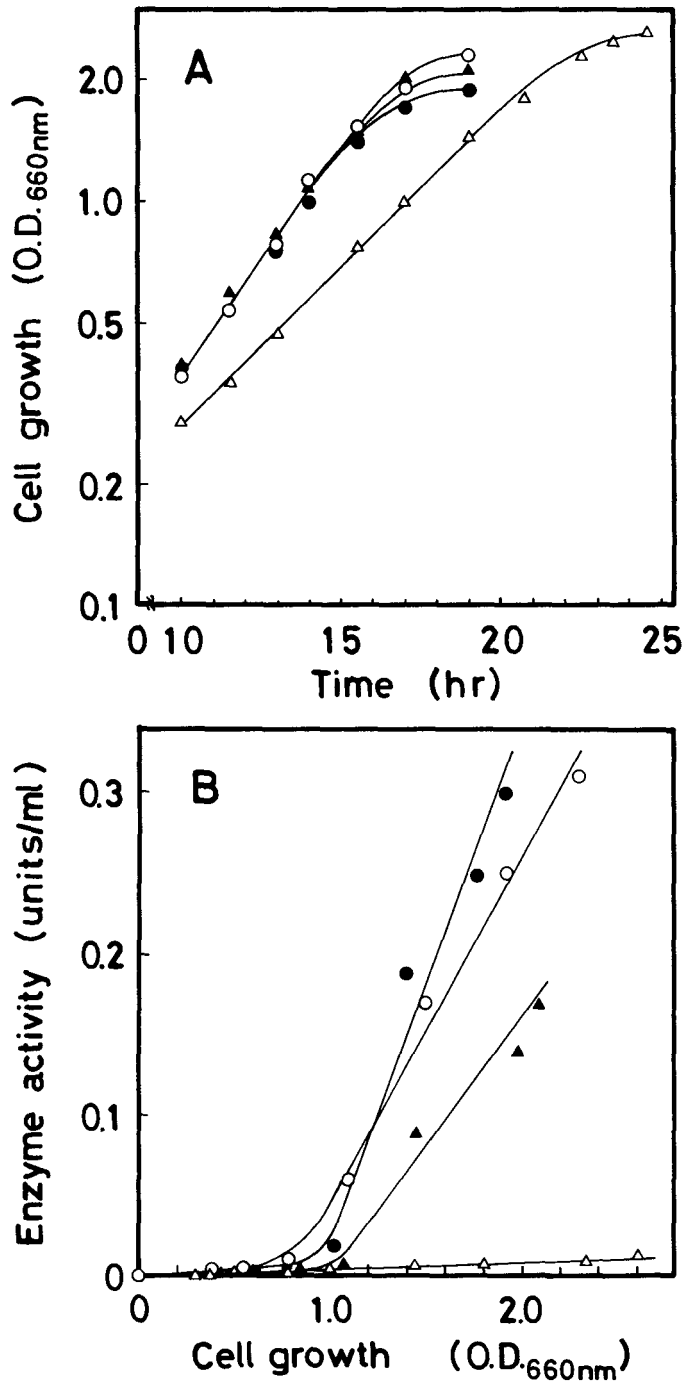

FIGURE 2.—Time courses of growth and acid phosphatase synthesis in wild-type and *cyr1-2* mutant strains. Cells of strain AM148-1C (wild type, ○ and ●) and AM148-1D (*cyr1-2*, △ and ▲) were shaken at 25° for 24 hr in YPGlu medium. Cells were harvested, washed and suspended in the same volume of sterilized water. A, A portion of the cell suspension was inoculated into 100 ml of low-P_i (○, △) or low-P_i·cAMP (●, ▲) media at the initial OD_{660} of about 0.05 and shaken at 25°. B, Acid phosphatase activity as a function of cell growth (OD_{660}) was determined by using an intact-cell suspension as the enzyme source.

when the mutant cells attained the same level of stationary growth as that of wild-type cells after prolonged incubation in low-P_i medium without cAMP.

The *cyr1-2* strain (AM148-1D) was grown in low-P_i medium at 25°. When the culture reached an OD_{660} of 1.6, cAMP was added to the culture, and the culture was divided into two parts. Cycloheximide (final concentration, 1 μg/ml) was added to one of the two parts. After 5 hr of incubation, the acid phosphatase activity in the absence of cycloheximide was increased from 0.004 to 0.058 unit/ml. The increase in acid phosphatase activity by addition of cAMP was not observed in the presence of cycloheximide.

The Pho^- phenotype of *cyr1-2* is similar to that of *pho2*, *pho4* and *pho81* mutations, which confer the inability to derepress acid phosphatase. *pho2* and *pho4* have been recently mapped on the left arm of chromosome *IV* and the right arm of chromosome *VI*, respectively (TOH-E 1980); *pho81* is located on the right arm of chromosome *VII* (A. TOH-E, personal communication). On the other hand, we found that *cyr1* was located on the right arm of chromosome *X* (MATSUMOTO *et al.* 1982a). The *cyr1-2* mutation complements *pho2*, *pho4* and *pho81* mutations with regard to the Pho^- phenotype on low-P_i medium at 25°, indicating that the *cyr1-2* mutation was different from these Pho^- mutations (data not shown).

Suppression of the Pho^- phenotype of cyr1-2 *mutants by the* bcy1 *mutation*: In our previous report, the *bcy1* mutant was found to bypass the *cyr1* defect without restoring the adenylate cyclase activity (MATSUMOTO *et al.* 1982a). The *bcy1* mutation results in the deficiency of cAMP-binding activity of cAMP-dependent protein kinase (Table 3; MATSUMOTO *et al.* 1982a). Strain AM9-8B (**a** *bcy1 pho3-1*) can be derepressed for acid phosphatase activity; addition of cAMP does not further increase acid phosphatase activity (Table 2). The result indicates that the *bcy1* mutation does not affect the derepression of repressible acid phosphatase.

Diploids constructed by crossing AM26-2A (**a** *cyr1-2 pho3-1*) to AM69-7D (α *bcy1 pho3-1*) were analyzed. Three ascus types showing 2+:2−, 3+:1− and 4+:0− segregations for growth on YPGlu medium at 35° appeared (five, seven and one asci, respectively); at 25° all 13 asci showed 4+:0− segregation on the same medium. Three ascus types showing 2+:2−, 3+:1− and 4+:0− segregations for the Pho phenotype on low-P_i medium at 25° were observed in a ratio of 5:7:1. Segregants that were able to grow at 35° showed the Pho^+ phenotype. One segregant from this cross, AM81-1C (**a** *cyr1-2 bcy1 pho3-1*), was crossed with AM104-1C (α *cyr1-2 pho3-1*), and diploids (AM105) obtained were subjected to tetrad analysis. Only one ascus type showing 2+:2− segregation for the Pho phenotype on low-P_i medium at 25° appeared in 12 asci. They showed 0+:4− segregation for the Pho phenotype on high-P_i medium at 25° and 2+:2− segregation for growth on YPGlu medium at 35° in all asci. Furthermore, tetrad clones of AM105-1 ascus were analyzed for acid phosphatase in low-P_i medium at 25° and examined for adenylate cyclase activity, cAMP content and the amount of cAMP-binding protein in YPGlu medium at 25°. Table 3 indicates that all segregants produced similar levels of adenylate cyclase activity and cAMP, but two *cyr1-2 bcy1 pho3-1* segregants (AM105-1A and D) produced no cAMP-binding activity and derepressed acid phosphatase.

TABLE 3

Acid phosphatase activity, adenylate cyclase activity, cAMP level and photoactivated binding of 8-N_3-[^{32}P]cAMP of tetrad segregants from AM105-1 ascus obtained from the AM81-1C × AM104-1C cross

Strain	Relevant genotype	Low-P_i	YPGlu		
		Acid phosphatase (units/$OD_{660\,nm}$)	Adenylate cyclase (units/mg protein)	cAMP level (pmol/mg protein)	8-N_3-[^{32}P]-cAMP bound (pmol/mg protein)
AM105-1A	*cyr1-2 bcy1 pho3-1*	0.09	0.3	0.5	<0.01
AM105-1B	*cyr1-2 + pho3-1*	<0.01	0.3	0.4	0.47
AM105-1C	*cyr1-2 + pho3-1*	<0.01	0.2	0.5	0.49
AM105-1D	*cyr1-2 bcy1 pho3-1*	0.12	0.2	0.5	<0.01

These results indicate that the *bcy1* mutation suppressed simultaneously the two phenotypes of the *cyr1-2* mutation, the inability to grow in the absence of cAMP and to derepress acid phosphatase.

To test effects of the *bcy1* mutation on mutations at the regulatory genes, *pho2*, *pho4* and *pho81*, diploids constructed by *bcy1* × *pho2*, *bcy1* × *pho4* and *bcy1* × *pho81* crosses were subjected to tetrad analysis. All asci obtained from these crosses showed a 2+:2− segregation pattern for derepression of acid phosphatase at 30°. The results indicate that the *bcy1* mutation does not suppress the inability to produce acid phosphatase activity of *pho2*, *pho4* and *pho81* mutants.

Acid phosphatase production by strains carrying the CYR3 *mutation*: *CYR3* mutants are able to grow on YPGlu medium at 25° but require cAMP for the best growth at 35°. The cAMP-dependent protein kinase activity of *CYR3* mutants shows significantly higher K_a values for activation by cAMP than those of wild type (UNO, MATSUMOTO and ISHIKAWA 1982). The apparent K_a of the wild-type enzyme was 0.04 μM, but that of the mutant enzyme was 0.20 μM at 25° and 2.00 μM at 35°. If the production of repressible acid phosphatase activity is dependent on the activation of protein kinase, it is expected that the acid phosphatase activity of *CYR3* mutants would be lower than that of wild-type strains. As shown in Table 2, AM33-1A (**a** *CYR3-1 pho3-1*) failed to derepress acid phosphatase activity at 25° but produced a significant level of acid phosphatase activity by addition of cAMP. When diploids constructed by the AM33-1A × G435-5B (α *CYR3+ pho3-1*) cross were subjected to tetrad analysis, 4+:0− segregation for growth on YPGlu medium at 25° and 2+:2− segregation at 35° were observed for seven asci examined. Five of seven tetrads were randomly selected, and the Pho phenotype and growth characteristics of segregants were determined (Figure 1). All *CYR3-1* segregants in seven asci showed no enzyme activity on low-P_i medium at 25°.

Effects of constitutive mutations for the repressible acid phosphatase synthesis on the cyr1-2 *mutation*: *pho80*, *PHO81^c*, *PHO82*, *PHO83* and *pho85* mutants produced acid phosphatase constitutively (Table 5). To investigate effects of these constitutive mutations on the *cyr1-2* mutation which is unable to derepress acid

TABLE 4

Tetrad segregations in crosses between strains carrying cyr1-2 *and phosphatase regulatory mutations*

Cross	Diploid strain	Relevant genotype of diploid	Segregation[a] in asci					
			Low-P_i			High-P_i		
			4+:0−	3+:1−	2+:2−	2+:2−	1+:3−	0+:4−
KYC168 × AM110-4D	AM139	$\frac{\alpha\ pho80\text{-}3\ pho3\text{-}1}{\mathbf{a}\ cyr1\text{-}2\ pho3\text{-}1}$	4	1	4	9	0	0
KYC188 × AM110-4D	AM140	$\frac{\alpha\ PHO81^c\ pho3\text{-}1}{\mathbf{a}\ cyr1\text{-}2\ pho3\text{-}1}$	2	8	2	12	0	0
P178-6C × AM110-4C	AM141	$\frac{\mathbf{a}\ PHO82\text{-}7\ pho3\text{-}1}{\alpha\ cyr1\text{-}2\ pho3\text{-}1}$	3	11	4	4	11	3
KYC95 × AM110-4C	AM142	$\frac{\mathbf{a}\ PHO83\ pho3\text{-}1}{\alpha\ cyr1\text{-}2\ pho3\text{-}1}$	0	0	14	2	7	5
KYC146 × AM110-4D	AM143	$\frac{\alpha\ pho85\ pho3\text{-}1}{\mathbf{a}\ cyr1\text{-}2\ pho3\text{-}1}$	4	6	3	13	0	0

[a] Each tetrad segregant was tested for its ability to produce acid phosphatase on low-P_i and high-P_i media by the staining method.

phosphatase, they were crossed with AM110-4C (*α cyr1-2 pho3-1*) or AM110-4D (**a** *cyr1-2 pho3-1*). The resultant diploids were sporulated, and four-spored asci were dissected. Segregants from each cross were cultivated at 25° on low-P_i or high-P_i medium, and their acid phosphatase activity was tested by staining (Table 4). In diploids prepared from KYC168 (*α pho80-3 pho3-1*) × AM110-4D, KYC188 (*α PHO81^c pho3-1*) × AM110-4D, and KYC146 (*α pho85 pho3-1*) × AM110-4D crosses, more than two segregants per ascus could synthesize acid phosphatase on low-P_i medium and there were always two constitutive spores per ascus, indicating that the *cyr1-2 pho80-3 pho3-1*, *cyr1-2 PHO81^c pho3-1* and *cyr1-2 pho85 pho3-1* mutants are constitutive for enzyme production. In diploids prepared from P178-6C (**a** *PHO82-7 pho3-1*) × AM110-4C (*α cyr1-2 pho3-1*), more than two segregants per ascus could produce acid phosphatase on low-P_i medium but less than two segregants per ascus were constitutive, suggesting that *cyr1-2 PHO82-7 pho3-1* segregants produced the enzyme in low-P_i medium but not in high-P_i medium. In diploids prepared from KYC95 (**a** *PHO83 pho3-1*) × AM110-4C, 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, indicating that *cyr1-2 PHO83 pho3-1* segregants did not produce acid phosphatase in either low-P_i or high-P_i medium.

The mutant strains, *cyr1-2 PHO80-3 pho3-1*, *cyr1-2 PHO81^c pho3-1*, *cyr1-2 PHO82-7 pho3-1*, *cyr1-2 PHO83 pho3-1* and *cyr1-2 pho85 pho3-1*, were obtained from these crosses and grown in low-P_i or high-P_i medium with shaking at 25°. The enzyme activity of each sample was determined by using intact cells as the enzyme source (Table 5). The mutants with *cyr1-2 pho80-3 pho3-1*, *cyr1-2 PHO81^c pho3-1* and *cyr1-2 pho85 pho3-1* genotypes showed reduced but significant levels of enzyme activity in both low-P_i and high-P_i media. Upon

TABLE 5

Acid phosphatase activity, adenylate cyclase activity and cAMP levels of mutant strains carrying cyr1-2 *and phosphatase regulatory mutations*

Strain	Relevant genotype	Acid phosphatase (units/$OD_{660\ nm}$)		YPGlu	
		Low-P_i	High-P_i	Adenylate cyclase (units/mg protein)	cAMP level (pmol/mg protein)
KYC168	+ *pho80-3 pho3-1*	0.29	0.29	4.7	1.9
AM139-5B	*cyr1-2 pho80-3 pho3-1*	0.07	0.08	0.3	0.2
KYC188	+ *PHO81*^{c} *pho3-1*	0.23	0.07	4.9	1.4
AM140-1C	*cyr1-2 PHO81*^{c} *pho3-1*	0.08	0.06	0.2	0.4
P178-6C	+ *PHO82-7 pho3-1*	0.25	0.02	3.4	1.8
AM141-1A	*cyr1-2 PHO82-7 pho3-1*	0.05	<0.01	0.2	0.5
KYC95	+ *PHO83 pho3-1*	0.08	0.04	8.8	1.9
AM142-5C	*cyr1-2 PHO83 pho3-1*	<0.01	0.01	0.2	0.5
KYC146	+ *pho85 pho3-1*	0.13	0.10	9.2	2.5
AM143-1C	*cyr1-2 pho85 pho3-1*	0.10	0.11	0.1	0.8

incubation in low-P_i medium, the *cyr1-2 PHO82-7 pho3-1* mutant showed low levels of enzyme activity, whereas it was repressed in high-P_i medium. The *cyr1-2 PHO83 pho3-1* was repressed for acid phosphatase in both low-P_i and high-P_i media. All of these mutants except *cyr1-2 pho85 pho3-1* could not grow on YPGlu medium at 35° but grew on YPGlu·cAMP medium at 35°. Since the *pho85* mutant could not grow on YPGlu medium at 35°, the *cyr1-2 pho85 pho3-1* mutant could not grow on both YPGlu and YPGlu·cAMP media at 35°. These results indicate that the constitutive mutants for acid phosphatase synthesis (except *PHO83*) suppressed partially the inability to produce acid phosphatase but did not suppress the inability to grow at 35° in strains carrying the *cyr1-2* mutation.

Effects of the cyr1-2 *mutation on synthesis of invertase and α-D-glucosidase*: To investigate whether the *cyr1-2* mutation affects the production of enzymes other than repressible acid phosphatase, the activity levels of invertase and α-D-glucosidase were investigated. To test invertase synthesis, tetrad clones of AM148-1 ascus prepared from AM110-4D × F10D were pregrown at 25° in YPGlu medium, transferred by YPGly plus 0.2% glucose at the initial OD_{660} of about 0.05 and harvested after 24-hr incubation in the same medium. To test clones for α-D-glucosidase production, cells grown in YPGlu medium at 25° were transferred to YPGlyMal plus 0.2% glucose at the initial OD_{660} of about 0.05 and harvested after 24-hr incubation. The cell density of each culture showed an OD_{660} of 1.0–2.0. As shown in Table 6, the *cyr1-2* segregants (AM148-1A and D) displayed significantly low levels of both enzymes as well as acid phosphatase at 25° in contrast to the wild-type segregants (AM148-1B and C) which produced high levels of the enzymes.

DISCUSSION

Strains carrying the *cyr1-2* mutation produced significantly low levels of

TABLE 6

Acid phosphatase activity, invertase activity and α-D-glucosidase activity of tetrad segregants from AM148-1 ascus obtained from the AM110-4D × F10D cross

Strain	Relevant genotype	Acid phosphatase (units/$OD_{660\ nm}$)	Invertase (units/$OD_{660\ nm}$)	α-D-glucosidase (units/$OD_{660\ nm}$)
		Low-P_i	YPGly + 0.2% glucose	YPGlyMal + 0.2% glucose
AM148-1A	*cyr1-2 pho3-1*	<0.01	0.30	0.03
AM148-1B	+ *pho3-1*	0.16	6.22	0.45
AM148-1C	+ *pho3-1*	0.13	5.38	0.40
AM148-1D	*cyr1-2 pho3-1*	<0.01	0.25	0.02

adenylate cyclase and cAMP and showed markedly reduced levels of repressible acid phosphatase, invertase and α-D-glucosidase under conditions that permitted growth of *cyr1-2* in the absence of cAMP. The addition of cAMP to the culture medium of this mutant led to the production of repressible acid phosphatase. From these results cAMP is implicated to be involved in the derepression of these enzymes. Since the *cyr1-2* mutants showed reduced growth rates in YPGlu medium at 25°, there might be a possibility that the *cyr1-2* mutant showed a deficiency in enzyme derepression due to the inadequate phosphate starvation or due to the reduction of general protein synthesis. This, however, is unlikely because (1) acid phosphatase was not derepressed even when the *cyr1-2* mutant cells attained the same level of stationary growth as that of wild-type cells after prolonged incubation in the absence of cAMP (Figure 2), (2) the *cyr1-2 PHO3+* strain produced almost the same level of constitutive acid phosphatase controlled by the *PHO3* gene as that of the *CYR1+ PHO3+* strain (Table 2) and (3) constitutive mutations for repressible acid phosphatase synthesis suppressed the inability to produce the enzyme but did not suppress the inability to grow at 35° in the *cyr1-2* mutants. Production of invertase and α-D-glucosidase was repressible by glucose (MATSUMOTO, TOH-E and OSHIMA 1981), but the synthesis of repressible acid phosphatase was not repressed by glucose. Therefore, it is not likely that the *cyr1-2* mutants may be defective in derepression of glucose-repressive synthesis of the enzymes in response to low concentrations of glucose.

The *bcy1* mutation suppressed the *cyr1* mutation without restoring the adenylate cyclase activity and resulted in the deficiency of regulatory subunit of cAMP-dependent protein kinase producing a high level of catalytic subunit of this enzyme (MATSUMOTO *et al.* 1982a). The *bcy1* mutation suppressed the inability to produce repressible acid phosphatase in the *cyr1-2* mutant. The increase of acid phosphatase activity by the addition of cAMP in low-P_i medium was not observed in this mutant. This is explained by our finding that the cAMP-dependent protein kinase of the *bcy1* strain is cAMP independent (MATSUMOTO *et al.* 1982a). *CYR3* mutants were unable to derepress acid phosphatase, but the addition of cAMP to the culture was effective in inducing acid phosphatase activity. This result is explained satisfactorily by our previous

finding that cAMP-dependent protein kinase of *CYR3* grown at 25° is structurally altered and requires exogenous cAMP to activate the mutant enzyme (UNO, MATSUMOTO and ISHIKAWA 1982). All of these results indicate that the production of repressible acid phosphatase is dependent on activation of cAMP-dependent protein kinase by cAMP.

The *cyr1* mutant differs from other mutants (*pho2*, *pho4* and *pho81*) that are unable to derepress acid phosphatase. The *bcy1* mutation could not suppress the Pho$^-$ phenotype of these mutations. The mutants carrying *cyr1-2* mutation and the *pho80*, *PHO81^c*, *PHO82* or *pho85* mutations, which confer constitutive synthesis of repressible acid phosphatase, produced repressible acid phosphatase. Recently, BOSTIAN *et al.* (1980) observed that derepression of acid phosphatase is the result of *de novo* appearance of functional mRNA followed by *de novo* protein synthesis. The participation of some of the *PHO* genes, including *PHO2*, *PHO4* and *PHO80*, in the regulation of *PHO5* transcription was further supported by KRAMER and ANDERSEN (1980). These authors detected mRNA in the *pho80* mutant regardless of whether cells were grown in high-P_i or low-P_i medium. On the basis of their studies, we speculate that the cAMP-dependent protein kinase exerts its function at the transcriptional stage in the synthesis of repressible acid phosphatase, although there remains the possibility that it works at the posttranscriptional level. To confirm the transcriptional regulation of repressible acid phosphatase by cAMP-dependent protein kinase, further work is required to analyze the RNA transcription from the *PHO5* gene in the *cyr1-2* mutant.

Studies on chromatin structure and its relationship to transcriptional activity have revealed that active genes have a conformation different from that of inactive genes in higher eukaryotes (WEINTRAUB and GROUDINE 1976) and yeast (SLEDZIEWSKI and YOUNG 1982; BERGMAN and KRAMER 1983). Phosphorylation of chromosomal proteins has been implicated in determining the ultrastructure of chromatin and in regulating the availability of genetic sequences for transcription in many biological systems (ISENBERG 1979). If phosphorylative modification of chromosomal proteins is related to the regulation of specific gene expression, selective phosphorylative modifications of nuclear protein might be responsible for changes of gene activity. A possible explanation of these data is that phosphorylative changes of nuclear protein by the cAMP-dependent protein kinase may modify chromatin structure in such a way as to allow derepression of specific enzyme synthesis.

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