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Genetics

Molecular analysis of the DNA sequences involved in the transcriptional regulation of the phosphate-repressible acid phosphatase gene (*PHO5*) of *Saccharomyces cerevisiae*

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ABSTRACT **The expression of the *PHO5* gene of *Saccharomyces cerevisiae* is transcriptionally regulated in response to the level of inorganic phosphate present in the growth medium. We have identified, by DNA deletion analysis, the sequences (upstream activator sequences) that mediate this response. The sequence 5′ CTGCACAA$^{A}_{T}$G 3′ is present in two copies located within a 60-base-pair region. The presence of a single copy of the sequence is sufficient for the phosphate-mediated transcriptional response. In addition, a DNA fragment that contains two copies of this sequence will act to repress transcription of a *CYC1–lacZ* fusion when placed either upstream or downstream of the *CYC1* activator sequence.**

The transcriptional regulation of genes in *Saccharomyces cerevisiae* is mediated through sequences located one hundred to several hundred bases upstream to the coding sequences (1–5). In all instances, there is a sequence, termed an upstream activator sequence, that stimulates transcription and is often the site of positive regulation or activation. In several cases, the binding of positive *trans*-acting regulatory factors has been demonstrated for these sites (6–8). In fewer instances, there have been demonstrated sites for negative regulation or repression (9, 10). However, at the present time, it is not known how either of these positive or negative sites mediate the activation or repression of transcription and transmit this information to the promoter proximal DNA sequences to activate or inactivate transcription.

To approach these questions, we have developed a high-copy plasmid system to investigate the expression of the phosphate-repressible acid phosphate gene (*PHO5*) of *S. cerevisiae*. The synthesis of acid phosphatase is controlled by a complex but genetically well-defined gene control system (11). Experiments from several laboratories have demonstrated that the expression of *PHO5* is under transcriptional control involving the gene products of several other genes; those responsible for repression of gene expression in the presence of phosphate (P_i) and induction of *de novo* synthesis of acid phosphatase in low P_i (12, 13). Sequences at the 5′ end determine a precise nucleosome positioning along the inactive gene sequence (14). In this study, using DNA deletions, we localize the sequences responsible for the transcriptional activation of *PHO5* transcription in response to the concentration of P_i in the growth medium. Also, using *CYC1–PHO5–lacZ* gene fusions, we have demonstrated the presence of a "negative" factor involved in the repression of *PHO5* gene expression.

MATERIALS AND METHODS

Strains and Media. Strains of *S. cerevisiae* utilized in this study are listed in Table 1. Strains were grown in YCAD medium [yeast nitrogen base (6.7 g/liter)/Casamino acids (5.0 g/liter)/adenine (0.4 g/liter)/glucose (20.0 g/liter) (or raffinose where noted) supplemented with tryptophan (20 μg/ml)] or low-P_i YCAD medium prepared according to Rubin (15). Yeast transformations were carried out as described by Ito *et al.* (16) using LiCl. Bacteria were grown and plasmid DNA was isolated as described (17).

Table 1. Genotypes and phenotype of *S. cerevisiae* strains used in this study

Strain	Genotype	Phenotype
S288C	α, *MAL*, *GAL2*	Wild type
GG100-14D	α, *pho5*, *his3*, *ura3-52*, *trp1*	Recessive negative
YAT29-40	α, *pho80*, *ura3-52*, *trp1*, *leu2-3*, *leu 2-112*, *ade2*	Recessive constitutive

Plasmids. The plasmid pBS-627 contains the 627-base-pair (bp) *Bam*HI/*Sal* I fragment containing the 5′ flanking region of the *PHO5* gene, the sites of transcription initiation, and the amino-terminal amino acids of the protein, cloned into the *Escherichia coli* plasmid pUC19 (18). In two derivatives, p627-TX and p627-G, the unique *Tth*111I site and *Bst*EII site were converted to an *Xho* I site and *Bgl* II site, respectively, by digestion with the appropriate enzyme, filling the single-stranded ends with DNA polymerase I and recircularizing the plasmid with T4 DNA ligase in the presence of either *Xho* I or *Bgl* II DNA linkers. The plasmid p12UTT2 (see Fig. 1 for the structure of the wild-type plasmid p19UTT2) contains the *E. coli* plasmid pUC19, the yeast *URA3* selectable marker, the 2-μm circle autonomously replicating segment, the *CYC1* transcription terminator, and a 1.2-kilobase *Sal* I/*Pst* I fragment containing the remainder of the *PHO5* gene. Thus, the plasmid contains unique *Bam*HI and *Sal* I sites, allowing the analysis of deletions created within pBS-627 and its derivatives. pLG669Z, a *CYC1–lacZ* vector was obtained from L. Guarente (19). Derivatives either lacking the 430-bp *Xho* I fragment (p669-ΔXho) or with unique *Xho* I sites (p669-XP or p669-XD) were constructed in the laboratory.

DNA Deletion Analysis and DNA Sequencing. DNA deletions within pBS-627 or its derivatives were created by either of two methods. (*i*) pBS-627 was cleaved with appropriate restriction enzymes, the single-stranded ends were filled-in with DNA polymerase I, and the plasmid was recircularized with T4 DNA ligase. (*ii*) pBS-627 was cleaved with either *Tth*111I or *Cla* I and treated with BAL-31 for various time intervals. The purified DNA was subsequently treated with T4 DNA polymerase and recircularized with T4 DNA ligase in the presence of either *Xho* I linker (for the *Tth*111I-cleaved sample) or *Cla* I linker (for the *Cla* I-cleaved sample). Individual *E. coli* transformants were screened by restriction analysis and subsequently the deletion fragment was purified by polyacrylamide gel electrophoresis. The fragment was

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Abbreviation: bp, base pair(s).

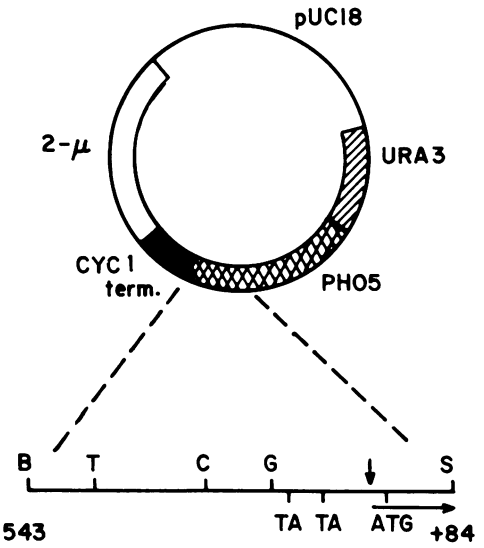

FIG. 1. Schematic representation of plasmid p19UTT2 containing the wild-type *PHO5* gene. B, *Bam*HI; T, *Tth*111I; C, *Cla* I; G, *Bst*EII; S, *Sal* I.

then cloned into p19UTT2 (see Fig. 1) cleaved with *Xho* I and *Sal* I (for deletions at the *Tth*111I site) or *Bam*HI and *Cla* I (for deletions at the *Cla* I site). The molecular nature of all DNA deletions was determined by DNA sequence analysis using the dideoxy method of Sanger and either M13 or direct sequencing from the plasmid DNA by using synthesized oligonucleotides corresponding to various regions of the PHO5 5′ flanking sequences (S.L.M. and L.W.B., unpublished observations).

Nomenclature. The base pairs in the plasmids are numbered with respect to the *PHO5* coding sequence. The A residue in the ATG initiation codon represents base pair 1; bases 3′ are numbered consecutively in positive integers, and bases 5′ are numbered consecutively in negative integers.

Acid Phosphatase and β-Galactosidase Assays. The measurement of acid phosphatase and β-galactosidase was essentially as described (14). One unit of acid phosphatase activity was taken as that liberating 1 μmol of *p*-nitrophenol per min. β-Galactosidase activity is expressed as nmol of *O*-nitrophenyl β-D-galactoside cleaved per min per mg of protein (20).

RESULTS

As illustrated in Fig. 1, we have developed a high-copy plasmid vector system that mimics the transcriptional regulation genomic copy of the *PHO5* gene. We have positioned the sequences responsible for the termination of transcription of the yeast *CYC1* gene immediately upstream (in the correct orientation) of the *PHO5* 5′ flanking sequences. This positioning in essence eliminates any complications that might arise due to read-through by RNA polymerase. The data for the wild-type plasmid (p19UTT2), as seen in Table 2, reveals an ≈60-fold increase in acid phosphatase enzyme activity under derepressed growth conditions (low P_i). This level is 4- to 5-fold higher than that seen in the wild-type strain S288C, which contains a single copy of the *PHO5* gene, and is consistent with the plasmid copy number of these transformants. Moreover, the level of acid phosphatase activity under repressed growth conditions (high P_i) is similar in the p19UTT2 transformant as compared to either strain S288C or the parent strain GG100-14D. In all deletion plasmids tested, there has been no effect on the level of acid phosphatase produced under repressed growth conditions.

Previous experiments using *PHO5–CYC1–lacZ* fusions have demonstrated that sequences responsible for the transcriptional regulation of the *PHO5* gene in response to the level of P_i are present on the 269-bp *Bam*HI/*Cla* I fragment (14). To further delineate the sequences responsible for this effect, we have created a series of DNA deletions in the 5′ flanking sequences of the *PHO5* gene by using combinations of various restriction enzyme digestions (see Fig. 1). The wild-type *PHO5* plasmid was digested with various enzymes, the single-stranded ends were filled in by DNA polymerase I, and the plasmids were recircularized with T4 DNA ligase. The nature of the DNA deletion was confirmed by DNA sequence analysis and, subsequently, strain GG100-14D was transformed with each plasmid. The cells were grown under repressed and derepressed growth conditions and the level of acid phosphatase was measured. As revealed in Table 2, deletion of the sequence −543 to −175 (ΔBG) or the sequences −444 to −175 (ΔTG) decreased the level of acid phosphatase produced under derepressed conditions by a factor of ≈10 (as compared to the level seen with the wild-type plasmid p19UTT2). The level of acid phosphatase produced in this strain is similar to that seen in the nontransformed host strain GG100-14D, suggesting that virtually all transcription of the plasmid-borne *PHO5* gene has been eliminated by this deletion. However, deletion of the 270-bp *Bam*HI/*Cla* I (ΔBC; −543 to −273) has caused only a partial reduction in acid phosphatase production, whereas a smaller deletion ΔTC (−444 to −273) has a slightly greater effect in reducing the level of acid phosphatase under derepressed growth conditions. Only deletion ΔBT (−543 to −444) had no effect on the production of acid phosphatase.

Deletion of the sequences −543 to −273, as discussed above, caused at least a 50% reduction in acid phosphatase

Table 2. Effect of DNA deletions on production of acid phosphatase

Strain	Plasmid*	DNA deletion	Enzyme activity,† units × 10^{-2}/OD_{600}		Relative activity‡	
			High P_i	Low P_i	High P_i	Low P_i
S288C	—	—	1.5	21.3	1.53	21.7
GG100-14D	—	—	1.4	8.8	1.43	9.0
GG100-14D	p19UTT2	—	1.6	98.2	1.63	100.0
	ΔBG	(−543 to −175)	1.7	10.9	1.73	11.1
	ΔTG	(−444 to −175)	1.4	12.5	1.43	7.6
	ΔBC	(−543 to −273)	1.6	45.2	1.63	46.1
	ΔTC	(−444 to −273)	1.3	22.8	1.32	23.2
	ΔBT	(−543 to −444)	1.6	94.1	1.63	95.8

*All plasmids were transformed into strain GG100-14D.
†Measurement of acid phosphatase is described in *Materials and Methods*.
‡The acid phosphatase activity in low-P_i conditions of strain GG100-14 transformed with the wild-type plasmid p19UTT2 is defined as 100 relative units. All other activities are determined relative to this standard.

Table 3. Synthesis of β-galactosidase in plasmid-transformed strain GG100-14D grown in low- and high-P_i media

Plasmid	Enzyme activity,* low-P_i/high-P_i ratio
pZ180X	190.8
pZ100X	1.6
pLG669Z	1.8
pLG669Z-ΔXho	1.3

*β-Galactosidase activity is described in *Materials and Methods*.

production. By using a derivative of pLG669Z that lacks the upstream activator sequences of the *CYC1* gene but retains the signals for transcription initiation fused to the *E. coli lacZ* gene, the presence of the *PHO5* upstream activator sequence on a DNA fragment that spans the sequences −444 to −273 (pZ180X) has been confirmed, as revealed in Table 3. The presence of this region causes an ≈200-fold increase in β-galactosidase activity under derepressed growth conditions, whereas the presence of sequences −543 to −444 (pZ100X) or the parent plasmid that lacks *PHO5* sequences do not show this effect. The level of derepression seen with plasmid (pZ180X) is consistent to that seen with the 270-bp *Bam*HI/*Cla* I fragment (−543 to −273) (14). Furthermore, by using this fusion plasmid, the effect of mutant *trans*-acting regulatory alleles that confer either high level constitutive synthesis (*pho80*) or a nonderepressed phenotype (*pho81, pho2, pho4*) is mediated through this DNA region (data not shown).

To further delineate the sequences involved in the regulation of *PHO5* expression, a series of DNA deletions was created by using the exonuclease activity of BAL-31 within the 170-bp region between the *Tth*111I and *Cla* I sites. The linear plasmid was treated with BAL-31 for increasing time periods, converted to blunt-ended molecules by using T4 DNA polymerase, and circularized with T4 DNA ligase in the presence of either *Xho* I linkers (for *Tth*111I digestion) or *Cla* I linkers (for *Cla* I digestion). Individual clones were screened by restriction digestion to determine the approximate size of the deletion. Subsequently, the DNA fragments were purified by polyacrylamide gel electrophoresis and cloned in the p19UTT2 vector cleaved with the appropriate enzymes. The molecular nature of each deletion was determined by DNA sequence analysis as described in *Materials and Methods*. As revealed in Fig. 2, deletions that originate at the *Tth*111I and proceed in the 3′ direction have no effect (including deletions to −407) until reaching deletion T11 (−444 to −389), where there is a partial reduction in *PHO5* expression. Deletion to −342 causes a further partial decrease; however, deletion of an additional 9 bp to −333 (T13) has effectively abolished *PHO5* transcription. Deletions that originate at the *Cla* I site proceed upstream to −370 (through the region implicated by the *Tth*111I deletions) with no effect on *PHO5* expression. Subsequent deletion of an additional 14 bp to −384 (C547) has completely blocked *PHO5* expression. This result suggests the presence of multiple upstream activator sequences and analysis of the DNA sequence within this region has revealed the conserved sequence 5′ CTGCACA$\frac{A}{T}$G 3′ located at residues −391 to −382 (UAS_I) and −341 to −332 (UAS_{II}). The deletions, as shown in Fig. 2, that block *PHO5* expression have removed either both of the regions (T14 or C581) or one region entirely and a portion of the second sequence (T13 or C547). However, deletion of a single conserved sequence appears to have no effect on the level of *PHO5* expression under derepressed conditions. None of the DNA deletions discussed in Fig. 2 has any effect on the level of *PHO5* expression under repressed conditions (data not shown).

To further investigate the molecular mechanism of *PHO5* expression—e.g., fully repressed in high-P_i growth medium and subsequently derepressed in low-P_i growth conditions, we have utilized the *CYC1–lacZ* plasmid system developed by Guarente (19). This fusion plasmid is expressed at low levels in cells growing on glucose as a sole carbon source and derepressed 3- to 6-fold in cells grown on a nonfermentable carbon source such as raffinose. The sequences responsible for the transcriptional regulation of the *CYC1* gene are located on a 430-bp *Xho* I fragment. Using *Xho* I linkers, we have positioned the 269-bp *Bam*HI/*Cla* I fragment, discussed previously, into either the promoter proximal *Xho* I site (termed pXP) such that the *PHO5* fragment lies between the *CYC1* UAS and the sites of transcription initiation or at the promoter-distal *Xho* I site (termed pXD) such that the inserted *PHO5* fragment is upstream of the *CYC1* UAS. Insertion of the *PHO5* fragment at either *Xho* I site in either orientation has blocked the expression of the *CYC1–lacZ*

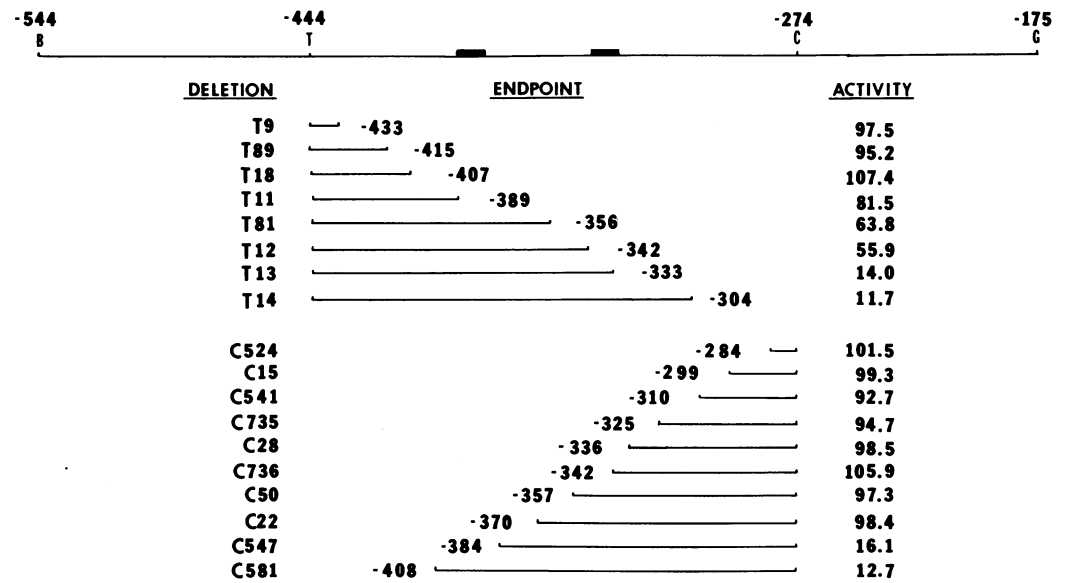

FIG. 2. Effect of BAL-31 DNA deletions within the 170-bp *Tth*111I/*Cla* I fragment on the relative synthesis of acid phosphatase under derepressed growth conditions (low phosphate). The level of synthesis in low phosphate of acid phosphatase in strain GG100-14D transformed with the wild-type plasmid p19UTT2 is defined as 100 relative units. The blocks present on the restriction map represent the location of the identified activator elements.

Table 4. Synthesis of β-galactosidase in plasmid-transformed strains GG100-14D and 29-40 (*pho80*)

Strain	Plasmid	Carbon source	Enzyme activity, units per mg of protein (high P_i)	Relative activity	Enzyme activity, units per mg of protein (low P_i)	Relative activity
GG100-14D	pLG669Z	Dex	3,872.5	100.0	3,980.7	102.8
		Raf	9,196.4	237.5	9,217.4	238.0
	pXP270A	Dex	408.2	10.5	10,370.5	267.8
		Raf	447.7	11.6	12,426.7	320.9
	pXD270A	Dex	692.3	17.9	11,485.3	296.6
		Raf	540.6	13.9	12,837.2	331.5
YAT 29-40	pLG669Z	Dex	3,526.2	91.0	3,216.0	83.0
		Raf	8,406.7	217.1	9,173.9	236.9
	pXP270A	Dex	10,726.8	277.0	10,015.4	258.6
		Raf	12,239.1	316.1	11,982.3	309.4
	pXD270A	Dex	10,112.2	261.1	10,534.9	272.0
		Raf	13,556.8	344.9	12,811.4	330.8

Dex, dextrose; Raf, raffinose.

fusion in glucose-grown cells and also repressed the increased level of *CYC1* expression in cells grown in raffinose, as seen in Table 4. Furthermore, the repression of *CYC1* expression is observed by using the 170-bp *Tth*111I/*Cla* I fragment containing the UAS_I and UAS_{II} regulatory sequences inserted at either *Xho* I site (pXP-170 or pXD-170) but not the 101-bp *Bam*HI/*Tth*111I fragment (pXP-100 and pXD-100) inserted at either site (data not shown). As seen in Table 4, the repression is reversed in strain YAT 29-40 containing the *pho80* mutation, which confers high-level constitutive synthesis on acid phosphatase. These results implicate the presence of a *trans*-acting regulatory factor that binds to the PHO5 DNA fragment, which subsequently blocks the interaction of the *CYC1* UAS region and the promoter proximal sequences important in initiation of *CYC1* transcription.

DISCUSSION

We have developed and used a high-copy plasmid system to analyze the DNA sequences involved in the transcriptional regulation of the *PHO5* gene in response to the level of P_i in the growth medium. Previous experiments by Brent and Ptashne have demonstrated that, although placement of the sequences responsible for the termination of transcription of the yeast *CYC1* gene between the upstream activator sequences of the *GAL10* gene and the promoter proximal sequences has blocked the induction of transcription upon growth in galactose, placement of the transcription terminator upstream of the *GAL* UAS had no effect on induction of transcription (20). Therefore, we have placed the *CYC1* transcription terminator immediately upstream of the *PHO5* 5′ flanking sequences to rule out complications due to read-through transcription present on the plasmid. Examination of the levels of acid phosphatase production under growth conditions that repress production of acid phosphatase have revealed (see Table 2) that there is no significant change in enzyme production under repressed growth conditions in the plasmid-transformed strain as compared to either the untransformed host (strain GG100-14D) or a wild-type strain (S288C). This low level of acid phosphatase production is due to expression of the "constitutive" *PHO3* gene (13).

Upon transfer to derepressed growth conditions, there is an ≈60-fold increase in acid phosphatase production, as measured by enzyme activity in the plasmid transformed strain. This increase is greater than that seen in the wild-type strain (S288C) and may reflect the increase in *PHO5* copy number in the plasmid-transformed strain. Previous experiments from Rogers and co-workers (13) and from our laboratory (21) have demonstrated that multiple copies of the *PHO5* gene appear to be regulated in a normal manner within the cell.

Previous experiments using *PHO5–CYC1–lacZ* fusions have demonstrated that DNA sequences that mediate the transcriptional response under derepressed conditions are present on a 269-bp *Bam*HI/*Cla* I fragment (14). However, deletion of fragments from the 5′ flanking sequences in our plasmid construction have yielded a varying degree of reduction of *PHO5* transcription, which ranged from an ≈50% reduction in *PHO5* expression (pΔBC, −544 to −273) to a 90% reduction (essentially the level seen in the *pho5* host strain GG100-14D) for pΔBG (−544 to −171) and pΔTG (−444 to −141) (see Table 2).

In all plasmids examined, deletion of the region between the *Tth*111I site and the *Cla* I site (−443 to −273) has decreased expression of the *PHO5* gene. Examination of mRNA levels by RNA blot analysis has shown that the decrease in enzyme level is due to the decrease of PHO5-specific mRNA (data not shown). Consistent with the central role of the sequences from −443 to −273, experiments utilizing this DNA fragment fused to the *CYC1* transcription initiation sequences and the *lacZ* gene have shown that this region is sufficient to mediate the response to the level of P_i (see Table 3). To examine the sequences responsible for regulation by the level of P_i, we have created a series of DNA deletions using BAL-31 that originate at either the *Cla* I site or *Tth*111I site within this region. The data in Fig. 2 reveal that deletions that span from the *Cla* I site at −273 through −370 have no effect on *PHO5* transcription, whereas deletion of an additional 14 bp to −384 has essentially completely blocked *PHO5* derepression. Deletions that initiate at the *Tth*111I site (−101) must proceed through the region implicated by the *Cla* I deletions until the end points of the deletions are at a minimum in the region near −344. These results suggest the presence of at least two regulatory sequence elements, and analysis of the DNA sequence has revealed the sequence 5′ CTGCACAA$\overset{A}{T}$G 3′ present at −391 to −382 (UAS_I) and −341 to −332 (UAS_{II}). The BAL-31 deletion data have suggested that one of these elements is alone sufficient for the transcriptional regulation but deletion of both elements blocks derepression of either completely. Deletion of UAS_I causes only a partial reduction in *PHO5* expression. Other yeast genes, such as *GAL1–GAL10* (2, 5), *CYC1* (4), and the genes under general amino acid control (1, 22) have shown multiple repeats of the sequences involved in transcriptional regulation. The *PHO5* sequence elements are at or near the region of DNase I hypersensitivity seen upon gene derepression (23). However, the relationship of these phenomena is not known at the present time. Interestingly, one of the identified sequence elements (UAS_I) is located

within an ≈70-bp internucleosomal region as determined by nucleosome mapping experiments (14). Experiments by Lohr have shown the UAS region of the *GAL1–GAL10* promoter to be non-nucleosomal in nature (24, 25).

In an attempt to understand the molecular interactions responsible for *PHO5* repression/derepression, we have assayed the effect of placing DNA sequences from the *PHO5* 5′ flanking sequences within the yeast *CYC1* gene. The *CYC1* gene is expressed at a low level in cells grown in glucose as a carbon source but derepressed 3- to 6-fold in cells grown on a nonfermentable carbon source such as raffinose. Placement of the 269-bp *Bam*HI/*Cla* I fragment or the 170-bp *Tth*111I/*Cla* I either between the *CYC1* UAS and the promoter region or immediately upstream of the *CYC1* UAS acts to repress the basal level of *CYC1* expression and blocks the derepression of *CYC1* transcription upon growth in raffinose in high phosphate, as seen in Table 4. This result suggests the interaction of a *trans*-acting factor with the *PHO5* sequences, which in this experiment blocks *CYC1* expression. Ptashne and co-workers have shown that the interaction of the *lexA* repressor with the *lexA* operator, when the repressor is expressed in yeast cells, blocks the induction of the yeast *GAL1* gene if the operator segment is placed between the UAS_{GAL} and the promoter region. However, different from *PHO5*, the *lexA* system does not block *GAL* induction if placed upstream of UAS_{GAL} (26). Similarly, Struhl has postulated that glucose repression of the *GAL* system is mediated via a negative regulatory factor in experiments in which the UAS_{GAL} represses expression of the constitutively expressed *HIS3* gene if placed upstream of the *HIS3* promoter (27).

The presence of the bound factor may facilitate the previously reported specific positioning of the nucleosomes within this region (14, 23). The observed repression by the PHO5 DNA fragment of *CYC1*-mediated transcription is relieved by the high-level constitutive *pho80* mutation, ruling out the effect being related to the inserted DNA fragment itself (see Table 4). At the present time, we are "footprinting" the *PHO5* upstream region to obtain information concerning the mechanism of *PHO5* gene expression. Two models exist that explain the observed results. (*i*) Bound to the UAS region of the repressed *PHO5* gene is a positive regulator factor but in an unmodified form, such that it will not promote transcription. Dimethyl sulfate protection experiments have shown that, presumably, the *GAL4* molecule is bound to the UAS_{GAL} even under conditions where transcription does not occur (7). Furthermore, under conditions of glucose repression, the UAS_{GAL} region still appears to be non-nucleosomal (25). Both genetic and recent biochemical experiments have demonstrated that regulation factors required for acid phosphatase expression are synthesized constitutively (11, 28). (*ii*) A negative regulator factor is bound to the *PHO5* upstream region under repressed conditions, which prevents the binding of the positive factors. The binding site for this factor may be the same or different than that of the positive factor. It is interesting to note that a portion of the *PHO5* UAS_I (−391 to −382), which appears to be located between two nucleosomes (14), may exist in a 9-bp perfect stem loop, possibly preventing binding of the positive factor. Currently S1 nuclease sensitivity experiments are under way to investigate possible changes in DNA structure within this region under conditions of repression or derepression. Further experimentation may provide additional evidence for either model.

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