

Regulated expression of a human interferon gene in yeast: Control by phosphate concentration or temperature

(repressible acid phosphatase/expression vector/yeast genetics)

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ABSTRACT The promoter/regulator region from the yeast repressible acid phosphatase gene was used to construct a vector for the regulated expression of cloned genes in yeast. The gene for human leukocyte interferon was inserted into this vector. Yeast cells transformed with the resulting plasmid produced significant amounts of interferon only when grown in medium lacking inorganic phosphate. Mutants in two acid phosphatase regulatory genes (coding for a defective repressor and a temperature-sensitive positive regulator) were used to develop a yeast strain that grew well at a high temperature (35°C) but produced interferon only at a low temperature (23°C), independent of phosphate concentration.

The expression of several cloned mammalian genes in the yeast *Saccharomyces cerevisiae* has been reported (1-3). For two cases (1, 2), the expression was constitutive because the genes were linked to the promoter region from the unregulated alcohol dehydrogenase I gene. Sometimes, however, regulated synthesis of a foreign gene might be desirable, such as in the case of a product that is toxic to the yeast cells. Toward this end, the promoter from the yeast phosphoglycerate kinase gene was used to direct the synthesis of a cloned human leukocyte interferon gene with the level controlled by the carbon source (3). While the "induced" level of expression was high, the "uninduced" level was also moderately high, resulting in an induction of only about 20-fold.

To develop a vector for highly regulated expression of foreign genes in yeast, we have used the 5'-flanking region from the yeast repressible acid phosphatase (APase) gene, *PHO5* (formerly *PHOE*) (4, 5). Transcription of the *PHO5* gene is tightly repressed when inorganic phosphate (P_i) is present in the growth medium but is induced to a high level when P_i is depleted (6, 7). By using a DNA fragment carrying the putative promoter/regulatory region from *PHO5*, a unique restriction enzyme site was introduced between the transcription start site and the translation initiator codon. The *PHO5* promoter expression vector was then tested by inserting the cloned gene for human leukocyte interferon (rIFN- α D) (1) into the vector at this site.

Because P_i depletion might not be the most efficient or practical method for induction, especially for large-scale growth, we have used APase regulatory mutants (8, 9) to develop a yeast strain that induces APase and, therefore, genes in the *PHO5* promoter expression vector only in response to a low temperature. A number of regulatory genes for *PHO5* expression have been identified, including some required for repression in the presence of P_i and some required for induction in low P_i (8). At the nonpermissive temperature (35°C) the cells grow well but produce no APase even in low- P_i medium, whereas at the permissive temperature (23°C) APase is synthesized with or without P_i in the medium. We have used this strain, carrying our *PHO5* pro-

motor expression vector, to demonstrate temperature-regulated expression of the rIFN- α D gene in yeast.

MATERIALS AND METHODS

Yeast Strains and Media. W301-18A (α *ade2-1 leu2-3,112 trp1-1 can1-100 ura3-1 his3-11,15*) was obtained from R. Rothstein, A138 (α *PHO5-2 pho80-2*) was from P. Hansche (9), and R6-3A (α *pho4^{ts}*) was from A. Toh-e. Strains from this work are P1-22 (α *pho80 trp1 ade2 his3 leu2*), 29B5 (α *pho80 pho4^{ts} trp1 ade2 his3 leu2*), and 29A21 (α *pho80 trp1 ade2 leu2*).

High- P_i medium was YNB + CAA as described (1) with adenine and uracil added when required. No- P_i medium was UMD (6) plus uracil when required with no added P_i . Both lack tryptophan for selection of yeast transformants carrying the plasmid.

DNA and Plasmids. The *PHO5* gene was obtained from an 8-kilobase (kb) *EcoRI* restriction fragment shown to contain this gene (7, 10). The yeast/*Escherichia coli* shuttle vector, YRp7, was from R. Davis (11). The 0.56-kb *EcoRI* fragment carrying rIFN- α D on the plasmid pFRS36 (1) was obtained from Genentech. The yeast gene for glyceraldehyde-3-phosphate dehydrogenase was obtained by screening a yeast DNA library with the cloned chicken gene (12).

Construction of Recombinant Plasmids. Restriction endonucleases were from New England Biolabs and Boehringer Mannheim, and phage T4 DNA ligase and *E. coli* DNA polymerase (Klenow fragment) were from New England Biolabs. The enzymes were used as recommended by the suppliers. Synthetic *Bam*HI and *Eco*RI linkers were from Collaborative Research (Waltham, MA).

Growth and Analysis of Transformants Carrying rIFN- α D. Yeast transformations were performed as described (13). Cells with plasmid were grown at 30°C in high- P_i medium to a density of about 3×10^6 cells per ml ($OD_{600} = 0.5$), at which time half of the culture was removed and these cells were harvested by centrifugation, washed with sterile water, and resuspended in no- P_i medium. Growth was continued at 30°C and samples were removed at intervals of approximately 3 hr for measurement of cell density (OD_{600}) and APase activity (14) expressed as A_{420} units from a 30-min assay per 1 OD_{600} unit of cells. Cell extracts for RNA (15) and interferon (see below) analysis were also prepared.

For temperature-shift experiments, transformants were grown in high- P_i medium at 35°C to an OD_{600} of about 1. The culture was divided in half and one half was continued at 35°C while the other was grown at 23°C. Samples were removed from both at various times and prepared for analysis as described above.

Preparation of Cell Extracts and Interferon Assay. For each time point, approximately 10^8 cells were harvested by centrifugation and resuspended in 1 ml of 7 M guanidine-HCl/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride. An

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Abbreviations: APase, acid phosphatase; kb, kilobase(s); rIFN- α D, recombinant human leukocyte interferon D.

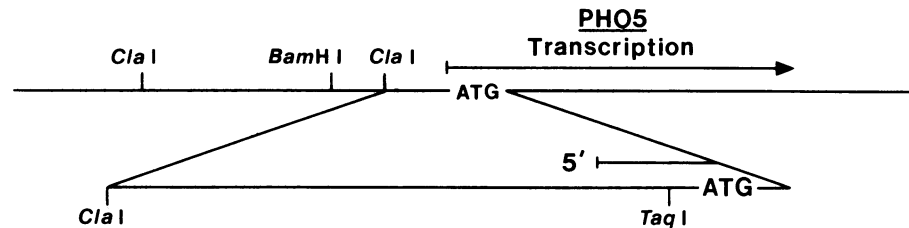


FIG. 1. Map of the *PHO5* 5' flanking region. The RNA 5'-end position (19) and the translation initiation codon as well as relevant restriction sites for the *PHO5* gene are shown. The *Taq* I site (at position -11 from the ATG) between the RNA 5' end and the ATG was converted to an *Eco*RI site for the expression vector. The major RNA 5' ends for *PHO5* transcripts map at positions -41 and -35 (19).

equal volume of acid-washed glass beads was added, and the samples were mixed on a Vortex mixer for four 30-sec bursts. After 30 min on ice, cell debris and glass beads were removed by centrifugation, and the samples were stored at -20°C . For interferon assay, the samples were either diluted 1:100 into 0.15 M NaCl/20 mM NaPO_4 , pH 7.9, or dialyzed against this buffer. Interferon activity was assayed by the reduction of cytopathic effect of vesicular stomatitis virus. Activities are expressed in units per liter of culture at an OD_{600} of 1.

RNA Gel Blotting and Hybridization. Total RNA from each time point was subjected to electrophoresis in 1.4% agarose gels after denaturation with glyoxal (17) and transferred to nitrocellulose (18). Hybridizations of the RNA blots were carried out as described (14) with ^{32}P -labeled DNA probes.

RESULTS

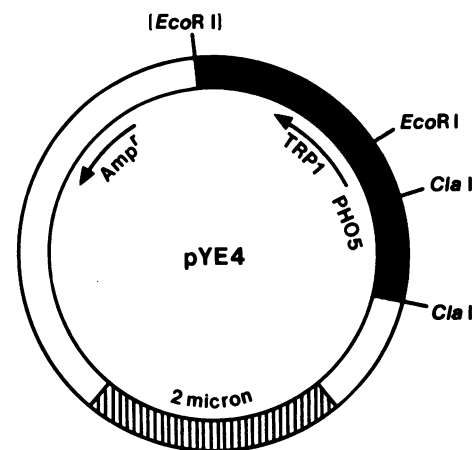
Construction of a *PHO5* Promoter Expression Vector. A unique restriction enzyme site was introduced into the DNA region between the position of the 5' end of the *PHO5* transcript (19) and the translation initiator ATG at the *Taq* I site shown in Fig. 1. This site was initially converted to a *Bam*HI site by "filling in" the sticky end with *E. coli* DNA polymerase Klenow fragment and ligation to the synthetic DNA fragment pC-C-G-G-A-T-C-C-G-G. To use the rIFN- α D gene with an *Eco*RI site preceding the initiation codon (1), this *Bam*HI site was converted to an *Eco*RI site by removing the *Bam*HI 5' overhang with S1 nuclease and ligation to the linker pG-G-A-A-T-T-C-C. The resulting 0.27-kb *Cla* I to *Eco*RI fragment with the transcription start site was inserted into the vector YRp7 (11), which carries the yeast *TRP1* gene on a 1.4-kb *Eco*RI restriction fragment in the plasmid pBR322 (20). The *Eco*RI site in parenthesis in Fig. 2 was removed from YRp7 as described (1). Because DNA sequences upstream from the *Cla* I site are required for full expression and regulation of the *PHO5* gene (unpublished observations; J. Lemire and K. Bostian, personal communication), the additional 1.1-kb *Cla* I fragment (Fig. 1) was also inserted. Finally, the plasmid was opened at the *Pvu* II and *Nru* I sites in the pBR322 portion (20), and a 1.88-kb *Nru* I/*Hinc*II fragment from the yeast 2- μ m plasmid (21), B form, was inserted. This fragment has the origin of replication and one inverted repeat (21) from the yeast plasmid. The resulting plasmid, pYE4, is diagrammed in Fig. 2A. A cloned gene carrying an ATG initiation codon can be inserted into the single *Eco*RI site in pYE4. Phosphate-regulated transcription will initiate in the *PHO5* promoter, proceed through the inserted gene, and terminate either in the 3'-untranslated region of the inserted DNA or at the termination site for the *TRP1* gene.

The expression in yeast of rIFN- α D had been previously reported with the yeast alcohol dehydrogenase I promoter (1), so this gene was used to test pYE4. The 0.56-kb rIFN- α D *Eco*RI fragment was inserted into pYE4, and a plasmid carrying the rIFN- α D gene in the correct orientation for expression from the *PHO5* promoter, designated pYE4-D, was obtained. Fig. 2B compares the nucleotide sequences of

the 5' noncoding regions of the original *PHO5* gene and of the *PHO5*/rIFN- α D fusion in pYE4-D. The extra C-C-G-G sequence in pYE4-D between the former *Taq* I site (T-C-G-A) and the *Eco*RI site (G-A-A-T-T-C) is from the synthetic linkers.

Regulation of Interferon Synthesis by Phosphate Concentration. Tryptophan-independent transformants of W301-18A carrying pYE4-D were grown in high- P_i medium at 30°C to an OD_{600} of about 0.5, at which time half of the culture was transferred to no- P_i medium. At intervals of approximately 3 hr, aliquots of each culture were removed and assayed for APase activity and extracts were prepared for interferon assay. Fig. 3 shows that the interferon activity began to increase rapidly between 3 and 6 hr after transfer to no- P_i medium and reached a maximum at around 9 hr. This induction corresponded to the APase induction observed for the culture and represented a 100- to 200-fold increase over the interferon activity detected in cells grown in high- P_i medium. In the example shown, the final level was 1×10^7 units per liter per OD_{600} for the induced culture and 6×10^4 for the

A



B

PHO5 AAGCAAATTCGAGATTACCA ATG
pYE4-D AAGCAAATTCGCCGGAATTC ATG

FIG. 2. pYE4 expression vector. (A) The *Cla* I to *Eco*RI fragment with the *PHO5* transcription start site was inserted into the yeast/*E. coli* shuttle vector YRp7 (11) that had the *Eco*RI site in parentheses deleted. The gene to be expressed under *PHO5* type regulation is inserted at the remaining *Eco*RI site. The arrow indicates the direction of transcription of the inserted gene and the yeast *TRP1* gene. The *TRP1* transcription termination site is about 0.8 kb past the *Eco*RI site. See *Results* for a full description of pYE4. Open bar indicates pBR322 DNA, filled bar indicates yeast DNA, and hatched bar indicates 2- μ m plasmid DNA. Amp^r, ampicillin resistance. (B) Nucleotide sequences preceding the translation initiation codon ATG in the original *PHO5* gene and in the *PHO5*/rIFN- α D fusion.

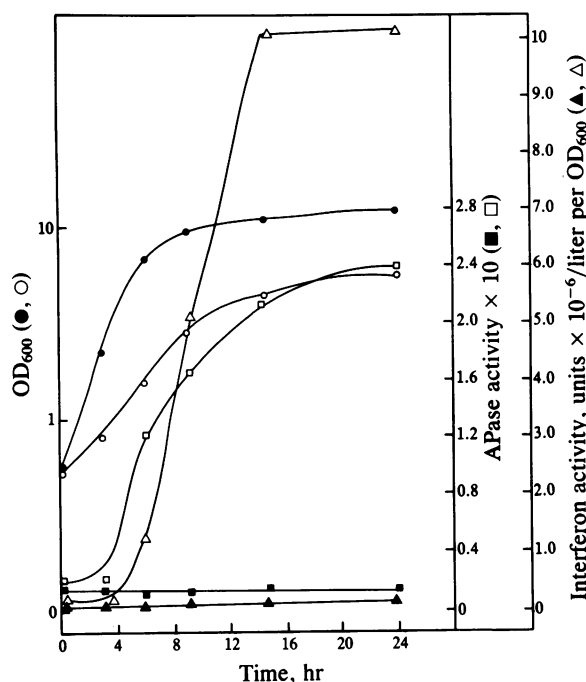


FIG. 3. Regulated expression of rIFN- α D in yeast. Transformants carrying pYE4-D were grown in high- P_i (solid symbols) or no- P_i (open symbols) medium. The cell growth (\bullet , \circ), APase activity (\blacksquare , \square), and interferon activity in extracts (\blacktriangle , \triangle) were monitored. Zero is the time at which the starter culture was divided into high- P_i or no- P_i medium.

uninduced. In other experiments, final levels of $2\text{--}3 \times 10^7$ were obtained.

RNA samples were prepared from both cultures at the same times that the interferon extracts were made. After denaturation with glyoxal (17), the RNA was fractionated on an agarose gel and then transferred to nitrocellulose (18). Fig. 4 shows the results of hybridization of the RNA blot with labeled rIFN- α D probe. The appearance of rIFN- α D-specific transcript corresponds to the appearance of interferon activity observed in the extracts. Thus, the regulation of interferon synthesis seen here is through RNA levels, as is the regulation of APase (6, 7). In addition, the size of the rIFN- α D-specific RNA is about 1.5 kb, the size expected for a transcript starting in the *PHO5* promoter, going through the rIFN- α D gene, and terminating at the end of the *TRP1* gene. In fact, the use of a different transcription termination region gave the expected RNA of a different size (not shown) than the one shown here. This proves that the RNA shown here is indeed from the interferon gene in the plasmid.

Regulation of Interferon Synthesis by Temperature. To construct a yeast strain capable of temperature-regulated APase induction, it was first desirable to eliminate the requirement for induction by low- P_i medium. Yeast mutants in the *PHO80* (formerly *PHOR*) gene, a repressor of APase synthesis, produce APase constitutively (9, 23). Therefore, a *pho80* strain (A138) was crossed with the *trp1* strain (W301-18A) used in the above experiments. A *pho80 trp1* strain (P1-22) was obtained and shown to produce APase in high- P_i medium. P1-22 was then crossed with a strain with a temperature-sensitive mutation in the *PHO4* (formerly *PHOD*) gene (R6-3A). *PHO4* codes for a positive regulator of APase synthesis (23) and, therefore, *pho4* strains are unable to produce APase even in low- P_i medium. A temperature-sensitive *pho4* strain can be induced by APase only at the permissive temperature.

A *pho4^{ts} pho80 trp1* host (29B5) was transformed with pYE4-D, and transformants were tested for temperature-regulated interferon synthesis. Cells were grown at 35°C to an

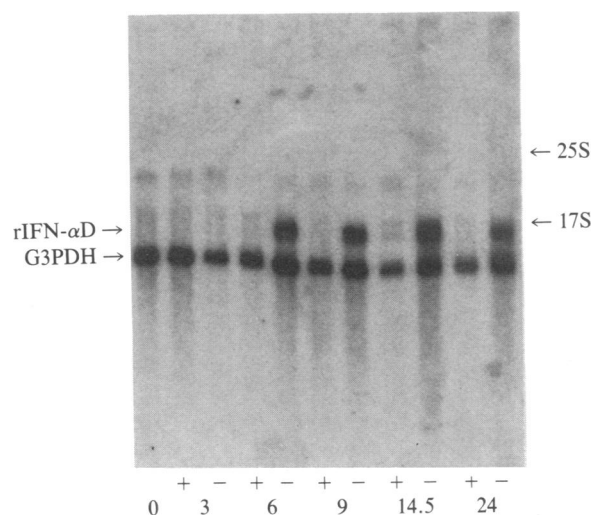


FIG. 4. Interferon RNA levels. RNA samples were prepared from the cells described in Fig. 3 (+ = high- P_i , - = no- P_i) at the indicated times (hr) and analyzed by gel blot analysis (18). Hybridization probes were 32 P-labeled DNA from the rIFN- α D gene and from the yeast glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene as a control. Glyceraldehyde-3-phosphate dehydrogenase is expressed at a high level in yeast (22) and should not be significantly affected by the level of P_i . The positions of the yeast ribosomal RNAs are also indicated.

OD₆₀₀ of about 1, and the culture was split into two portions. One continued to grow at 35°C, while the other was shifted to the permissive temperature (23°C). At intervals of approximately 2 hr, aliquots were removed and prepared for interferon assay. Fig. 5 shows that interferon activity was at least 50 times higher in the cells grown at 23°C. A final level of about 3×10^6 units per liter per OD₆₀₀ was reached in some experiments.

DISCUSSION

We have used the promoter/regulatory region from *PHO5*, the yeast repressible APase gene, to construct a vector

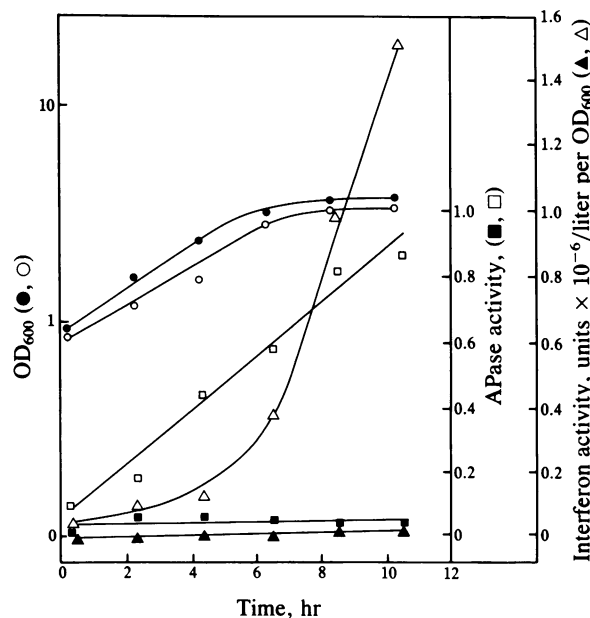


FIG. 5. Temperature regulation of interferon synthesis. The *pho80 pho4^{ts}* strain 29B5 transformed with pYE4-D was grown at 35°C to an OD₆₀₀ of 1, at which time (0) half the culture was shifted to 23°C (open symbols) and the other half was kept at 35°C (solid symbols). Cell growth (\bullet , \circ), APase activity (\blacksquare , \square), and interferon activity (\blacktriangle , \triangle) were monitored.

(pYE4) for tightly regulated foreign gene expression in yeast. More than 300 base pairs of DNA sequences upstream from the initiation codon were required for full expression and regulation. Because a unique restriction enzyme site was created in the DNA region corresponding to the 5' noncoding portion of the APase mRNA (19), the transcription start site for *PHO5* remains, but the entire coding region, including the initiator ATG, has been removed. Thus, a gene inserted into this site will have translation started at its own initiator codon, resulting in an authentic rather than a fusion protein.

When the gene for a human leukocyte interferon was inserted into pYE4, interferon activity was detected in yeast transformants, and the interferon synthesis was shown to be under *PHO5* type control. Thus, the elements involved in APase regulation appear to be present in sufficient amounts to also regulate the plasmid-carried *PHO5* promoter. This is in agreement with the finding of Rogers *et al.* (10) that the *PHO5* gene itself is correctly regulated when present on a multicopy plasmid.

Levels of up to $2-3 \times 10^7$ units of interferon activity per liter of culture at an OD_{600} of 1 were obtained in several induction experiments. While this is somewhat higher than that reported for the same gene with the yeast alcohol dehydrogenase I promoter (1), an even higher level might be expected from the amount of rIFN- α D-specific RNA observed (Fig. 4). There appears to be approximately the same level of rIFN- α D RNA as that for glyceraldehyde-3-phosphate dehydrogenase. This glycolytic enzyme accounts for about 5% of the total cellular protein in yeast (22), while the interferon activity observed here (assuming a specific activity of 2×10^8 units/mg for rIFN- α D) corresponds to a yield of about 0.2% of the protein. Thus, the level of interferon activity obtained with pYE4-D does not appear to be proportional to the high level of the RNA.

The nucleotide sequence of the 5'-untranslated region of the *PHO5*/rIFN- α D fusion is shown in Fig. 2B. Comparison of sequences for highly expressed yeast genes and foreign genes expressed efficiently in yeast (24) suggests that the presence of multiple G residues immediately preceding the initiation codon may significantly decrease translation efficiency in yeast. Thus, the C-C-G-G-G stretch resulting from the linker insertion could be the cause of the apparent inefficient translation reported here. Other factors such as protein or message instability or codon usage problems (24) might also affect the level of expression.

As an alternative to induction by P_i starvation, a *pho80 pho4^{ts}* strain (29B5) was constructed and used to demonstrate temperature-regulated interferon synthesis. Significant interferon activity is observed only at the permissive temperature, and the induced level reaches a maximum about 2 times higher than the final point in Fig. 5—i.e., about 3×10^6 units per liter per OD_{600} . This is about 1/5th to 1/10th of that observed for no- P_i induction and could be due to the somewhat poorer growth of the 29B5 at 23°C compared to W301-18A at 30°C (Figs. 3 and 5) or the possible lack of full *PHO4* activity of the particular *pho4^{ts}* mutation used here. Transformants of pYE4-D in a strain with the *pho80* mutation but not the *pho4^{ts}* (29A21) obtained in the same cross as 29B5 synthesized approximately the same amount of interferon in high- P_i medium at 30°C as the no- P_i -induced strains.

Finally, note that the temperature regulation reported here involves induction by a shift to a lower temperature rather

than to a higher temperature. A potential advantage of the shift-down system would be for the production of relatively unstable proteins that might be degraded more rapidly at high temperatures.

During the preparation of this manuscript, the expression of hepatitis B virus surface antigen in yeast with the *PHO5* promoter was reported (25).

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