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Isolation, physical characterization and expression analysis of the *Saccharomyces cerevisiae* positive regulatory gene *PHO4*

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ABSTRACT

The Saccharomyces cerevisiae PHO4 gene, which positively controls the expression of phosphatase genes, has been isolated by complementation of a pho4 mutation. The isolated DNA directed integration at the chromosomal PHO4 locus. The nucleotide sequence of PHO4 has a coding region of 930 nucleotides, flanked by sequences with typical transcription initiation and termination signals. The 5' region has characteristics of low-expression promoters and carries several uncommon elements, whose significance is not known. The predicted primary structure of the PHO4 protein, of 309 residues, does not show sequence elements typical of DNA-binding proteins. The transcription of PHO4 is independent of inorganic phosphate. Like other regulatory genes, PHO4 is transcribed at a very low level and the translation of its message uses preferentially several codons which are not employed for highly expressed genes.

INTRODUCTION

Considerable progress has been made recently in the comprehension of the molecular mechanisms by which transcription is regulated at gene upstream sites in the yeast Saccharomyces cerevisiae (reviews by Guarente (1) and by Brent (2)). There are currently several well investigated cases of positive control of yeast structural genes, where both cis- and trans-acting elements have been identified and shown to mediate activation of gene transcription, in response to specific physiological signals. DNA segments carrying cis-activating sites, usually called UAS (Upstream Activating Sequence) in yeast, have been delimited and sequenced. Their most striking feature, by which they are similar to many mammalian enhancer sequences, is their flexibility with respect to orientation and distance (one to several hundreds of bp) from the transcription initiation sites.The trans-acting elements were usually identified by earlier classical genetic work and it was presumed that their gene products exert their positive effects on gene expression by interacting or influencing interactions with gene upstream sites. Specific binding of regulatory proteins to gene upstream regions,that are critical for transcriptional activation, has been

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demonstrated recently in yeast for the galactose system and the system of general control of amino-acid biosynthesis : the GAL 4 protein binds to a UAS adjacent to the galactose inducible GAL 1 and GAL 10 genes (3,4); the GCN 4 protein binds to specific promoter regions of amino acid biosynthetic genes whose transcription is activated in response to amino acid starvation (5).

The present study deals with the yeast positive regulatory gene PHO4 whose product is presumed to interact with UASs upstream of several phosphatase encoding PHO genes. Yeast produces several constitutive and phosphate-repressible acid and alkaline phosphatases (APase and AlkPase, respectively). The repressible enzymes are under the control of inorganic phosphate (Pi) through a complex regulatory circuit. Genetic studies (6), data on the levels of the products of PHO genes (7-11) and molecular analysis of the regulated and highly expressible APase-encoding PHO5 gene (12-16); Legrain et al., in preparation) indicate that regulation of the repressible phosphatases occurs at the transcriptional level and it strongly suggests the following mechanisms. Transcription of the APase genes is activated by a process that requires the interaction of the PHO2 and PHO4 products (activators) with UASs upstream of the regulated structural PHO genes. Activation of the regulated AlkPase gene requires the action of the PHO4 activator only. The availability of the PHO2 product for activation of the APase genes is independent from the Pi effector. The availability of the PHO4 activator is controlled by Pi through the products of 3 genes : PHO80, PHO81 and PHO85. When Pi is present, the PHO4 activator is bound by the PHO80 and PHO85 products and is prevented to activate the structural PHO genes. In the absence of Pi, the PHO4 activator is released through the mediation of the PHO81 product.

Here we report the isolation and sequencing of a DNA segment that carries the PHO4 gene. We discuss several features of its flanking regions as well as expected properties of the predicted PHO4 protein. We also report data on the PHO4 transcript levels and comment about peculiarities of codon usage in PHO4 translation.

MATERIAL AND METHODS

Strains

E. coli : HB101 (r_k^-, m_k^+, leu, pro, recA) and JM105 (Δpro-lac, thi, $strA^r$, RsdR4, endA, sbcB, F'traD36, proAB, $lacI^q$, lacZΔM15).

S. cerevisiae : 1278b (α ; wild-type) ; 3962c (a ; wild type) ; ML39a

(α,pho4, ura3) was constructed by crossing strain YAT42 (α , pho3, pho4, arg6), obtained from Dr. A. Toh-e, with the well transformable yeast strain 02026c (a, ura3), obtained from Dr. E. Dubois.

Vectors

Plasmid vector pFL1 has been described by Chevallier et al. (17). It consists of a chimeric bacterial-yeast plasmid containing E. coli pBR322, part of the yeast 2μ plasmid and the yeast URA3 gene. The phage vectors used for sequencing were M13mp18 and M13mp19(18).

Yeast gene library

The yeast gene library, a gift from Dr. E. Dubois, was prepared by inserting yeast DNA fragments, produced by partial digestion of total DNA from the wild-type strain 1278b with Sau3A, into the BamHI site of pFL1.

Media

YEPD medium contained 2 % Bacto Peptone, 1 % Bacto Yeast Extract and 2 % glucose. Yeast minimal medium contained 3 % glucose, 0,1 % asparagine, vitamins and mineral traces ; it was prepared as described by Messenguy (19) with the following modification : high Pi medium contained 1.5g/l KH_2PO_4 and low Pi medium contained 0.03g/l KH_2PO_4 plus 1.5g/l KCl ; uracil, when necessary, was added at 50 μg/ml. LB medium (0.5% Yeast Extract, 1 % Bacto Tryptone, 1 % NaCl) contained 50 μg/ml ampicillin, when used to propagate plasmid harboring E. coli HB101. E. coli JM105, used for propagation of phage vectors, was grown on YT medium (0.5 % Yeast Extract, 0.8 % Bacto Tryptone, 0.5 % NaCl).

Genetic analysis

Genetic analysis on yeast was performed by standard methods (20).

Transformation

E. Coli HB101 cells were transformed according to Petes et al. (21), except that after growth in LB medium for 60 min. at 37°C, the cells were plated directly on selective medium, omitting suspension in soft agar. Transformation of E. coli JM105 with phage DNA was performed as described by Messing (22).

Yeast cells were transformed according to Hinnen et al. (23), but glucuronidase/arylsulfatase (from Boehringer, Mannheim, FRG) was used instead of glusulase to generate protoplasts and regeneration medium was the selective minimal medium containing 3 % agar and 1M sorbitol.

DNA preparation

Plasmid DNA was purified from E. coli according to the method described by Godson and Vapnek (24), followed by ethidium bromide/CsCl equilibrium

centrifugation. Single-stranded template was prepared as described by Messing (22). Rapid yeast DNA preparation was made according to Struhl et al. (25).

DNA sequence determination

E. coli JM105 was transformed with phage M13 DNA after insertion of PHO4 restriction fragments into the appropriate replicative form M13mp18 or M13mp19 (18,22). Sequencing was carried out using the dideoxynucleotide chain termination method (26).

Detection of acid phosphatase activity

Acid phosphatase activity in yeast colonies was performed by the staining method based on the diazo coupling reaction (27,28) by overlayering them with staining agar consisting of 1 % Difco agar, 0.1M acetate buffer pH4.0, 2mg/ml Fast Blue Salt B (Serva) and 0.2mg/ml α naphtyl phosphate (Serva).

Nothern blot analysis

RNA from Saccharomyces cerevisiae was purified according to Elder et al. (29). RNA enriched for molecules with polyadenylate sequences was prepared by a single passage over a oligodeoxythymidylate-cellulose column as described by Fraser (30). Electrophoresis, transfer and hybridization were according to Thomas (31).

Labelling

Nick translation of the DNA probes was performed following the procedure of Rigby et al. (32). The single stranded (ss) DNA was labelled by the technique used for the determination of the nucleotide sequence. Copies of the ss DNA were synthesized by the Klenow fragment of E. coli DNA polymerase using the universal primer, but without dideoxy nucleotides (33).

RESULTS

Cloning of genomic DNA sequences that complement the pho4 mutation

A pho4 ura3 (underepressible APase and uracil requirement) yeast strain was transformed with a DNA library of yeast genomic DNA fragments inserted into the BamHI site of the URA3 carrying vector pFL1 (Materials and Methods). Uracil-independent (Ura^+) transformants were selected by plating on medium lacking uracil. The Ura^+ colonies were then replica-plated on low Pi medium and screened for high APase activity by a colony staining procedure (Material and Methods). Among approximately 10.000 Ura^+ transformants screened, two showed a high APase level. When these were tested on high Pi medium, their APase activity was very low. This phenotype is expected for transformants having acquired the pFL1

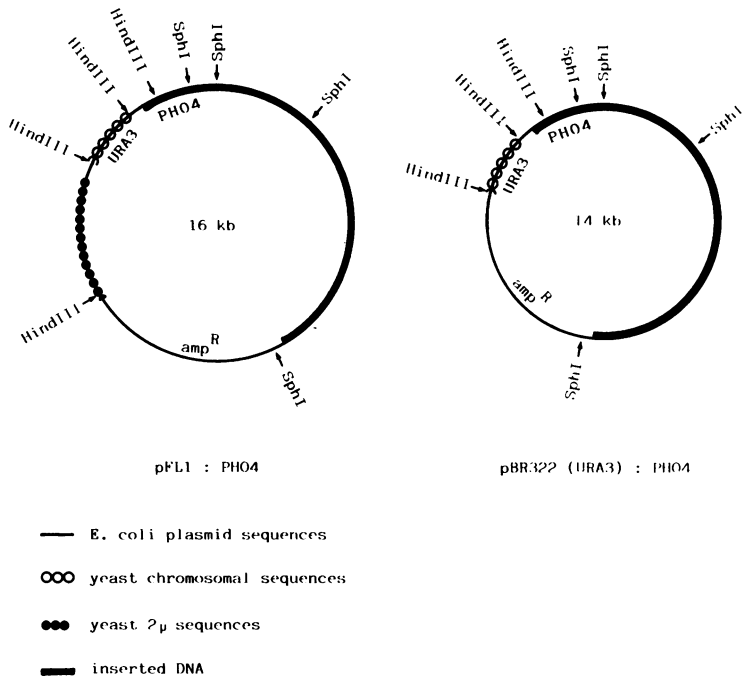

Fig. 1 Plasmids pFL1 : PHO4 and pBR322 (URA3) : PHO4.

plasmid with a PHO4 insert (Pho^+Ura^+ transformants).

DNA isolated from the 2 Ura^+Pho^+ candidates transformed E. coli to ampicillin resistance. The plasmids recovered from E. coli after amplification transformed pho4 ura3 yeast strains to Ura^+Pho^+. Restriction endonuclease mapping of the hybrid plasmids revealed that they carried identical, 8.3 kb inserts (Figure 1).

The regulated APase activity in the 2 selected Ura^+Pho^+ transformants was shown to be linked to the URA3 plasmid by a mitotic segregation test under non-selective growth conditions. The transformants were grown for 20 generations on YEPD ; cells were then plated out on the same medium and the colonies formed were replica-plated on low and high Pi medium, with and without uracil. The cosegregation of the Pho^+ and Ura^+ phenotypes was perfect, with 20 to 30 % of the clones having lost both characters.

The cloned fragment is derived from the PHO4 locus

The previous results strongly suggest that the cloned fragment carries the PHO4 gene. To confirm this, we determined the site of chromosomal

Table I.
Test for the integration of plasmid pBR322(URA3): PHO4 at the chromosomal pho4 locus.

	PHO4 : pho4 tetrad types (n°)		
	4 : 0	3 : 1	2 : 2
Expected if plasmid integrated at :			
- the pho4 locus	100	0	0
- a locus not linked to pho4	17	66	17
Observed :	16	1	0
	URA3 : ura3 tetrad types (n°)		
	4 : 0	3 : 1	2 : 2
Expected if plasmid integrated at :			
- the ura3 locus	100	0	0
- a locus not linked to ura3	17	66*	17
Observed :	3	12	2

Tetrad analysis was performed on the asci from the diploid ML39A pBR322 (URA3):PHO4 x 3962c (α, ura3 pho4 pBR322 (URA3):PHO4 x a, wild type). *This proportion would be lower, if the plasmid integrated close to a centromere.

integration directed by the cloned fragment. We constructed plasmid pBR322(URA3):PHO4 (Figure 1) which carries URA3 and the cloned 8.3 kb DNA fragment, but is devoid of a yeast replication origin. Such a plasmid would transform yeast at very low frequency by integrating into chromosomal sites homologous to plasmid-borne yeast DNA (34,35). From the extent of homology between the 2 possible integration sites (1.1 kb for URA3; 8.3 kb for the cloned insert), integration is expected to take place predominantly into or close to the pho4 locus. Yeast strain ML39a (α, ura3 pho4) was transformed with plasmid pBR322(URA3):PHO4 and a transformant was crossed with the wild-type strain 3962c. Tetrad analysis of the meiotic progeny from the resulting diploid lead to the results shown in Table 1. They show that the cloned segment integrated at a site tightly linked to the pho4 locus on chromosome VI, and that the cointegrated URA3 vector gene is unlinked to the ura3 locus on chromosome V.

Nucleotide sequence of the PHO4 region

Subcloning of the 8.3 kb DNA segment carrying PHO4 lead to the isolation of a 3.1 kb fragment (Figure 2) that still complemented the pho4 mutation.

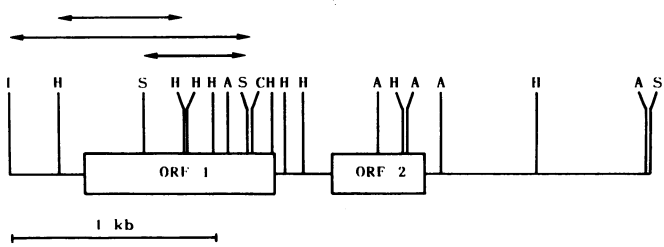

Fig. 2 Restriction map of the 3.1 kb fragment carrying the PHO4 gene.
A = Sau3A, C = ClaI, H = HpaII, I = HindIII, S = SphI; ORF = open reading frame. The arrowed lines indicate the restriction fragments used as probes in the Northern blot analysis.

Sequencing of this fragment revealed the presence of 2 open reading frames(ORF), 310 and 148 codons long, separated by a 256 bp non-coding region. By comparison of the predicted protein sequence of the smaller ORF with those published at this time it appeared that it is homologous, except for one bp, to the sequence encoding subunit VI of the ubiquinol-cytochrome C reductase (36 and Legrain et al., in preparation). That the larger ORF encodes the PHO4 function was supported by the finding that an inversion of the 480 bp SphI fragment (Figure 2) within the ORF lead to the inactivation of PHO4.

Both strands of the PHO4 gene were completely sequenced by the dideoxynucleotide chain termination method (26), except for two short stretches (100 and 110 bp) which were sequenced several times on the same strand. The sequencing strategy was as indicated in Figure 3. The DNA sequence is shown in Figure 4.

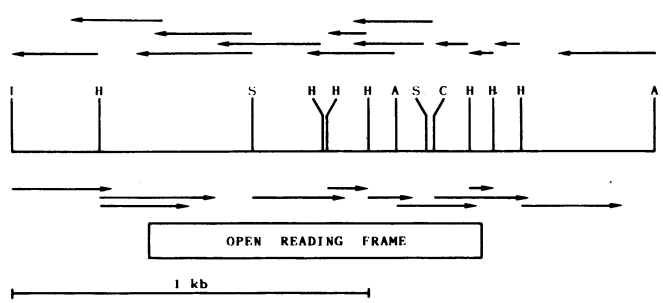

Fig.3 Strategy for the determination of the nucleotide sequence of PHO4 (read left to right as 5' to 3'). The arrows indicate the length and direction of the sequenced fragments.
A = Sau3A, C = ClaI, H = HpaII, I = HindIII, S = SphI

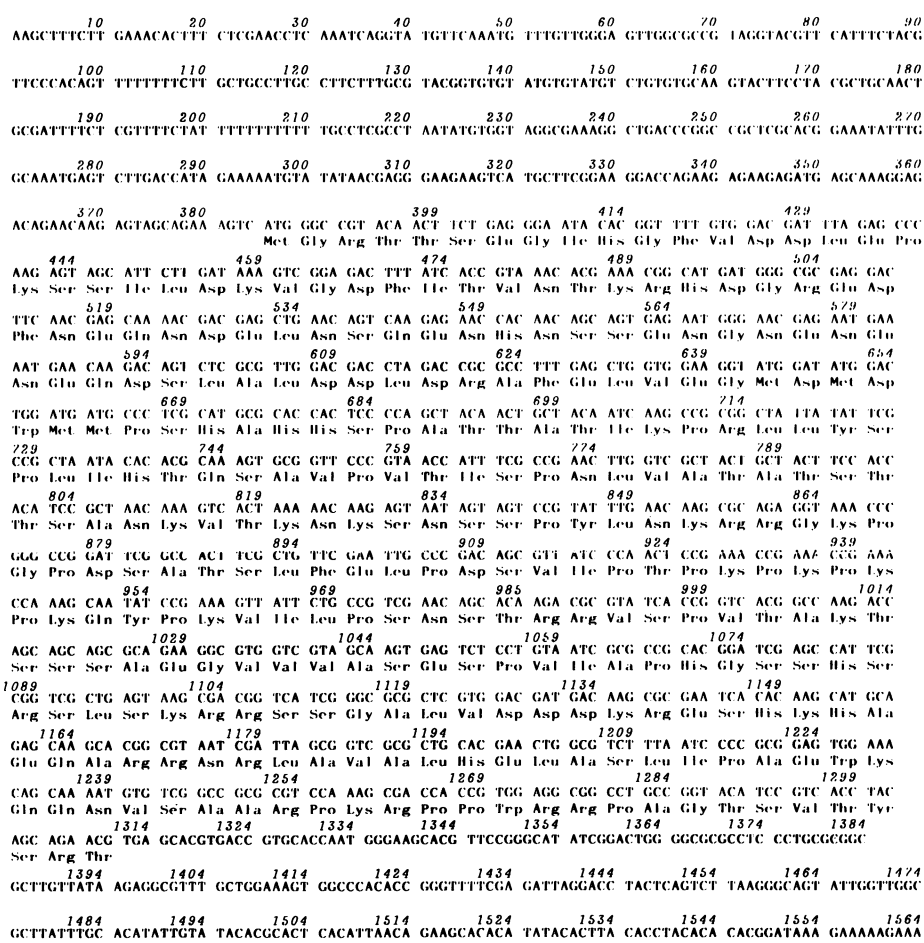

Fig. 4 Nucleotide sequence and deduced amino acid sequence of the S. cerevisiae PHO4 gene.

The largest ORF begins with an ATG triplet at position 385 and ends with the stop codon TGA at position 1314. Translation of the PHO4 message is probably initiated at the ATG at position 385, because this ATG fits best the features that are usually encountered for functional translation initiator codons in eucaryotic genes in general and in yeast genes in particular, i.e. the presence of typical promoter sequences immediately upstream of this ATG and of significant sequences around it (38-42; see discussion). The next in-frame ATG lies 256 bp further downstream. The ORF, delimited as indicated above, contains 930 nucleotides. This size is

Table II. Codon usage for the PHO4 gene.

		N° COD.	N° AA			N° COD.	N° AA			N° COD.	N° AA			N° COD.	N° AA
Phe	UUU	3		Ser	*UCU	3		Tyr	UAU	3		Glu	*GAA	7	
Phe	*UUC	2	5	Ser	*UCC	4		Tyr	*UAC	1	4	Glu	GAG	12	19
				Ser	UCA	3									
Leu	UUA	4		Ser	UCG	11			UAA	0		Cys	*UGU	0	
Leu	*UUG	4		Ser	AGU	10			UAG	0		Cys	UGC	0	0
Leu	CUU	1		Ser	AGC	9	40		UGA	1					
Leu	CUC	2										Trp	UGG	3	3
Leu	CUA	3		Pro	CCU	2		His	CAU	4					
Leu	CUG	7	21	Pro	CCC	6		His	*CAC	8	12	Arg	CGU	3	
				Pro	*CCA	5						Arg	CGC	5	
Ile	*AUU	3		Pro	CCG	13	26	Gln	*CAA	7		Arg	CGA	3	
Ile	*AUC	5						Gln	CAG	1	8	Arg	CGG	6	
Ile	AUA	2	10	Thr	*ACU	7						Arg	*AGA	3	
				Thr	*ACC	5		Asn	AAU	6		Arg	AGG	1	21
Met	AUG	5	5	Thr	ACA	6		Asn	*AAC	12	18				
				Thr	ACG	4	22								
								Lys	AAA	10					
Val	*GUU	3		Ala	*GCU	5		Lys	*AAG	10	20	Gly	*GGU	4	
Val	*GUC	7		Ala	*GCC	5						Gly	GGC	3	
Val	GUA	5		Ala	GCA	4		Asp	GAU	6		Gly	GGA	3	
Val	GUG	5	20	Ala	GCG	10	24	Asp	GAC	12	18	Gly	GGG	3	13

N° cod. = number of times each codon appears in the PHO4 coding region. N° aa = number of each amino acid in the PHO4 protein. * designates the codons which are preferentially used for highly expressed yeast genes, like ADH1 and G3PDH (44). The codons that are underlined are "rare" codons which are preferentially used in the PHO4 genes (see text).

consistent with that found for the PHO4 mRNA, which is about 1.5 kb (see below). The highly, if not strictly, conserved sequence elements for the pre-mRNA splicing in yeast (5'-GTATGT-3' at 5' splice sites and 5'-TACTAAC-3' within the 3' part of introns) are absent from the PHO4 region.

The PHO4 termination region contains sequences partially homologous to those proposed by Zaret and Sherman (43) to be involved in 3'-mRNA-end

formation in yeast (TAG...TACT or TATT...TTT, 124 to 168 bp after the stop codon). Sequences similar to other possible termination signals (44, 45) are not apparent.

The predicted primary structure of the PHO4 protein.

The amino acid sequence, read from the ORF which appears to correspond to the PHO4 coding region (Figure 4), contains 309 residues, i.e. a calculated molecular weight of 33,888 dalton. It contains no cys and the most abundant amino acids are ser (12,9%) and pro (8,4%). The distribution of several amino acids is far from uniform, especially for the acidic and basic residues. The moderately acidic NH_2-terminal region is followed by an extremely acidic segment between residues 41 and 90 (18 asp + glu versus 2 his + arg), which in its first part is also very rich in asn and gln (12 residues). Further downstream the protein becomes highly basic (from residues 96 to 161 : 13 arg + his + lys versus 0 asp + glu). 20 residues farther (180-193) appears a striking pro-lys alternation sequence : $(pro\text{-}lys)_4$-gln-tyr-pro-lys. The carboxyl-terminal part of the protein is also very basic.

The codon usage (Table II) is very typically that of genes with low level expression (44, 46). The codon bias index (CBI) is - 1.6 percent.

Transcription analysis.

We performed Northern blot analysis on RNAs from Pi-repressed and derepressed cells from strains with one and with several copies of PHO4 (Figure 6). With 3 different restriction fragments from the coding region as probes (Figure 2), we detected a single hybridization band, corresponding to an RNA of about 1.5 kb. That this RNA corresponds to the PHO4 transcript was confirmed by an hybridization experiment with the single strand prepared from one of the restriction fragments (Figure 6; Materials and Methods). It hybridized only to the strand complementary to the PHO4 coding sequence. The amounts of this RNA are approximately the same for the different culture conditions, which is evidence that the expression of PHO4 is constitutive. The experiment also reveals that the cellular concentration of the PHO4 transcript is extremely low (hybridization conditions in the legend of Figure 6 and in "Materials and Methods").

DISCUSSION

The 3.1 kb DNA fragment that we isolated from a yeast genomic library contains the PHO4 information, since it mediates complementation of a pho4

Fig. 5 Nucleotide sequence of the PHO4 5' non-coding region.
A. The complete sequence with the first coding triplet ATG. Regions showing dyad symmetry are underlined (the smaller elements) or overlined (the large element). The symmetry centers are indicated by a dot. The limits of the symmetry elements are indicated by arrows. The TATATAA sequence is boxed. Wavy lines underline the TC-rich and CAAG sequences, and overline a purine-pyrimidine alternation segment.
B. Comparison of the sequences immediately preceding the ATG codon for PHO4 and GAL4 (37). Blanks are used to maximize identity of the sequences, which are complete.

mutation and directs its integration at the chromosomal PHO4 locus. It carries a 930 bp open reading frame, which is closely flanked by typical transcription initiation and termination sequences, and which is devoid of splicing signals. Its integrity is necessary for the PHO4 function. Single- and double-stranded DNA segments from this region hybridize to a unique and rare RNA of 1.5 kb. We may therefore consider that the delimited coding region is colinear with the PHO4 protein.

The PHO4 promoter region presents several significant sequences (Figure 5). The 79 bp stretch between the typical TATATAA sequence and the translation start codon is very rich in A and G (61A+G/18C+T) and contains numerous repeats composed of these nucleotides. The 30 bp sequence preceding the PHO4 coding region shows striking homology with the corresponding region of GAL4, which is also a positive regulatory gene. The PHO4 sequence upstream from the "TATA box" contains 2 CT-rich blocks at positions 79-127 and 185-220. The first block is separated from a downstream CAAG motif by a segment of 30 bp which is an almost perfect Pu-Py alternation, with T being by far the predominant Py. The region between the second TC-rich block and a site 20 bp upstream of the "TATA box" contains numerous trinucleotide repeats (with G and C) and dyad symmetry elements. Several smaller symmetry elements are distinguishable,

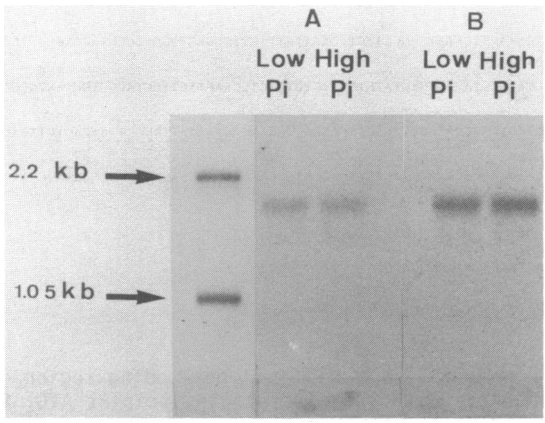

Fig. 6 Northern analysis of PHO4 mRNA.
Poly(A)-RNAs (~5ug) were fractionated on 1.4% agarose gel containing formaldehyde. After transfer to nitrocellulose, the RNAs were hybridized with a ^{32}P HpaII-HpaII (720 bp) single-stranded probe, labelled by primer extension (0,05µg DNA per ml of hybridization mixture at 5.10^7 cpm/ug). The X-ray films were exposed with an intensifying screen at -70°C.
A. Wild-type strain 1278b; film exposed for 20 days.
B. ura3 pho4 strain (ML39a), carrying plasmid pFL1-PHO4; film exposed for 3 days.
Molecular weight markers in lane on the left : pBR327 DNA digested with AvaI and BamHI.

but almost the whole region (60 nucleotides) may also be ordered around a single point (position 245) to constitute a structure with extensive two-fold rotational symmetry. Further studies should help to disclose what function, if any, the Pu-Py alternation sequence and the redundant repeats and symmetry elements have. As to the CT-rich blocks, associated or not with a CAAG motif, their lay-out has been related to the strength of gene expression (40) and the ordering of these elements in the PHO4 gene corresponds to that of low-expression genes.

The PHO4 protein is presumed to activate the transcription of several phosphatase genes and it is tempting to assign it a mode of action similar to that of the GAL4 protein, whose binding to a UAS upstream of GAL structural genes has been demonstrated recently (3.4). The PHO4 protein does not carry sequence elements with striking homology to sequences identified as essential for nuclear transport (47-49) or for DNA-binding (46), as they are found in the GAL4 protein. The PHO4 protein is however integrated in a regulatory network of higher complexity, as outlined in the "Introduction". It interacts with several other regulatory elements and, in

the activation process itself, the PHO2 protein probably plays a crucial role. Further insight into the specific role of the PHO4 element will probably come from the characterization of the PHO2 gene and its products, which is in progress (50).

The measurement of PHO4 mRNA levels at different growth conditions indicates that the expression of PHO4 is constitutive with respect to inorganic phosphate. As expected for a regulatory gene, the PHO4 mRNA level is extremely low.

A study on codon selection in yeast (44), for moderately to highly expressed genes, has evidenced a marked preference for 25 out of the 61 possible coding triplets. Moreover, in the cases where tRNA sequence data were available, it was found that, for a given amino acid, the major isoaccepting tRNA species present in yeast was, in fact, that with an anticodon allowing it to translate the most frequently used codon. The degree of usage of 22 preferred codons (indicated in Table II) has been quantitated by the Codon Bias Index (CBI), which is 1 when only the 22 preferred codons are used in a gene and which is 0 when the usage of these codons is exactly that expected if the code were read randomly. The same study shows that for 9 genes that are considered, the CBI is correlated with the level of total cellular mRNAs. Kammerer et al. (46) have calculated the CBIs for 13 other yeast genes, among which are 6 regulatory genes. The 4 lowest CBI values are for the regulatory genes GAL4, PPR1, MAT α2 and MATα1 with values of +3.6, -0.9, -2.3 and -3.4%, respectively. The value that we find for PHO4 is -1.6%. These close-to-zero CBIs would indicate an almost totally random codon usage in these regulatory genes. However, a distinct asymmetry of codon usage is apparent for several amino acids. If we consider those codons in PHO4, which are usually not used in highly expressed genes and whose usage is for instance 1.5 times that expected if the code were read randomly, 5 codons (underlined in Table II) are sorted out : CUG (leu), UCG (ser), CCG (pro), GCG (ala) and CGG (arg). These 5 "rare" triplets code for 15.2% of the total amino acids in the PHO4 protein. They would code for 8.5% of the total amino acids, if their usage were at random. tRNAs with anticodons complementary to these 5 codons are probably all minor species (not mentioned in the compilation of the major yeast isoaccepting tRNAs, by Bennetzen and Hall (44)). The great similarity, in their preference for "rare" codons, between PHO4 and the regulatory genes mentioned above, suggests that this codon usage pattern has functional significance. It will be interesting to see to what extent

the cellular protein and mRNA levels do correlate for genes with low expression, like PHO4, when they are artificially induced to high expression. It is finally worth noting that the 5 "rare" codons, preferred in PHO4, are all GC-rich codons and have all a G in the wobble position, which, according to the wobble rules in yeast (51), should pair exclusively with a C in the anticodons. These codons would thus have higher than average binding constants for their complementary anticodons (44) and, consequently, might allow a more efficient utilization of the cognate tRNAs from their low cellular pools.

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