
Negative regulators of the PHO system in *Saccharomyces cerevisiae*: isolation and structural characterization of *PHO85*

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ABSTRACT

One of the negative regulators of the PHO system of *Saccharomyces cerevisiae*, *PHO85*, has been isolated by transformation and complementation of a *pho85* strain. The complementing activity was delimited within a 1258 bp DNA segment and this region has been sequenced. The largest open reading frame found in this region can encode a protein of 302 amino acid residues. A *pho85* mutant resulted from disruption of the chromosomal counterpart of the open reading frame described above. Therefore, we concluded that the gene we have cloned is *PHO85*. This result also indicates that *PHO85* is nonessential. Northern analysis revealed that the size of the *PHO85* message is 1.1 kb. No similarity was found between the putative amino acid sequences of two negative regulators, the *PHO80* and *PHO85* proteins.

INTRODUCTION

A shortage of available inorganic phosphate in the medium leads to depression of several enzymes or proteins included in the PHO system of *Saccharomyces cerevisiae*; they are repressible acid phosphatases (1,2), repressible alkaline phosphatase (3) and phosphate permease (4). At least 5 regulatory genes have been identified in this system. *PHO2* and *PHO4* are positive regulators and *PHO80* and *PHO85* have negative functions. The *PHO81* gene product, which is hypothesized as a mediator (5), is believed to inactivate a negative factor(s). Recently, expression of *PHO81* was found to be controlled by the PHO system (6). According to our current model, positive factors play an essential role in the derepression of the structural genes. Negative factors exert their function by controlling the activity of positive factors at the protein level.

In order to examine the above model, it is necessary to characterize the regulatory proteins and the promoters of the structural genes whose expression is controlled by the PHO system. Two positive regulator genes, *PHO2* (7) and *PHO4* (8), have been cloned and sequenced. *PHO81* has been cloned and its expression was analysed (6). We have isolated one of the negative regulators,

PHO80 (9). The promoter of the PHO5 gene, which is one of the structural genes encoding repressible acid phosphatase has been extensively analysed and its upstream regulatory region defined (10,11,12). The PHO8 gene encoding repressible alkaline phosphatase has been cloned (13) and analysis of its promoter is underway (to be published elsewhere). In this communication, we describe the cloning and sequencing of another negative regulator, PHO85. The codon usage of the putative PHO85 gene product shows characteristics of a gene expressed at a low level. No similarity was found between the PHO80 and PHO85 sequences in spite of the phenotypic resemblance of their mutants.

MATERIALS AND METHODS

Strains and plasmids

Saccharomyces cerevisiae DC5 (MAT α PHO3 leu2 his3), which is a generous gift of J.N. Strathern, was used as DNA source for the construction of a yeast gene library in YCp19. An isogenic pair, YAT953 (MAT α leu2 his3 pho3) and YAT954 (MAT α leu2 his3 pho3), was used as the wild type strains. Transformants thereof were also used as described in the text. YAT1175 (MAT α leu2 trp1 ura3 pho3 pho85) was used as the host to clone the PHO85 gene.

Escherichia coli DHL (14) (F⁻ recA1 gyrA96 thi hsdR17 supE44 relA1) was used as a host strain in the construction and propagation of plasmids. JM103 (15) (Δ lac-pro supE thi strA endA hsdR4 sbcB15, F' traD36 proAB lacI^q Δ M15) was used for growing M13 phage and its derivatives. YCp19 (16), YIp5 (17), YIp32 (17), and YEp13 (18) were used as yeast vectors. M13mp18 and M13mp19 (15) were used for DNA sequencing. The yeast gene library was constructed by inserting DNA fragments of DC5 cells longer than 10 kb generated by partial digestion with Sau3AI into the BamHI site of YCp19.

Media

YEPD contained 2% Polypeptone (Daigo Eiyō), 1% Yeast extract (Oriental Yeast) and 2% glucose. High-Pi medium was prepared as described previously (1). SD contained 0.67% Yeast Nitrogen Base without amino acid (DIFCO) and 2% glucose. Nutrient broth, L-broth, and YT-broth were prepared as described (19) and used for bacterial cultivation. Tryptophan (20 μ g/ml), uracil (20 μ g/ml), Casamino acid (DIFCO) (0.5%), ampicillin (50 μ g/ml), and tetracyclin (10 μ g/ml) were added as appropriate. Solid media contained 2% agar.

Preparation of DNA

Yeast genomic DNA was isolated from the indicated strain by the rapid method (20). Plasmid DNA was isolated from E. coli according to the method described

by Birnboim and Doly (21). Plasmid DNA was purified by CsCl-Ethidium bromide banding as necessary.

Detection of acid phosphatase activity

Acid phosphatase activity of colonies was detected by the staining method described previously (22).

Transformation

Yeast transformation was done by the lithium acetate method (23). Competent cells of *E. coli* for transformation were prepared as described previously (24).

Isolation of Poly(A)⁺-RNA

Total RNA was extracted according to the method described by Jensen et al. (25). Poly(A)⁺-RNA was partially purified by oligo dT-cellulose column chromatography.

Determination of nucleotide sequences

DNA sequences were determined by the dideoxy chain termination method (26) using a sequencing kit provided by Takara Shuzo Co. DNA segments to be

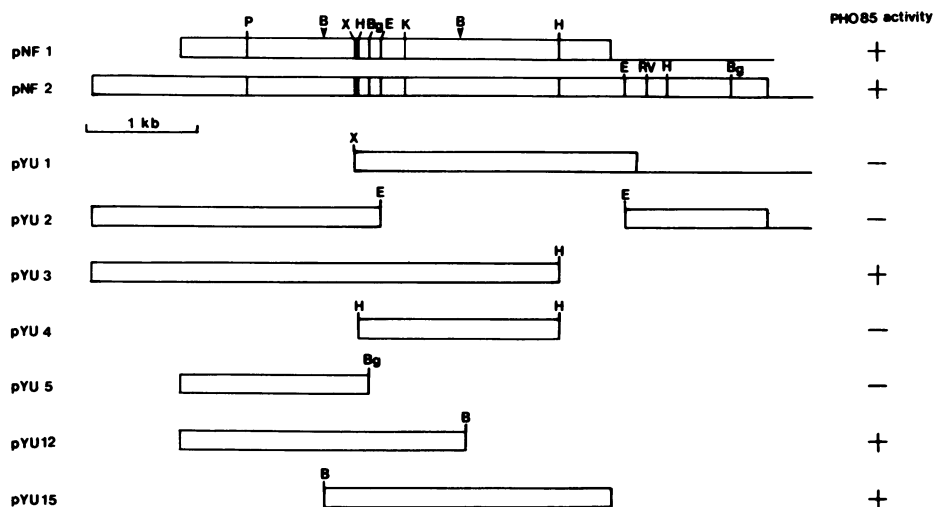


Fig. 1 Restriction map of the cloned DNA containing the PHO85 gene. Open rectangles indicate the region of the cloned DNA. Each plasmid containing the indicated fragment was constructed by deletion of pNF1 or pNF2 or by re-cloning the segment into the other yeast vectors. The resulting plasmid was introduced into the pho85 host to test whether it complements the pho85 mutation. ∇ , site insertion of a BamHI linker; B, BamHI; E, EcoRI; G, BglII; H, HindIII; K, KpnI; X, XhoI.

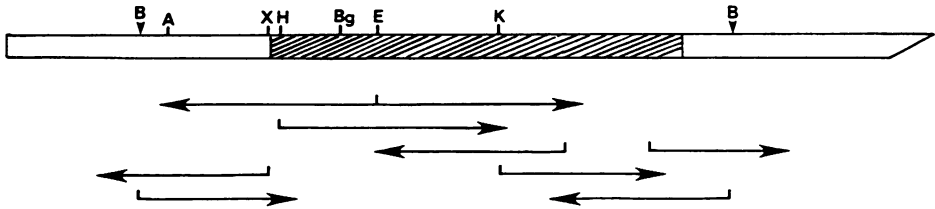


Fig. 2 Strategy for sequencing. Arrows indicate the location, direction, and length of each sequence. Hatched area indicates the largest open reading frame in this region. Symbols are the same as those used in Fig.1 except A, which indicates an *AccI* site.

sequenced were subcloned into a single-stranded DNA vectors, either M13mp18 or M13mp19 (15).

Linker insertion

A *Bam*HI linker was inserted randomly into plasmid pNF1 (See Fig.1) according to the method described by Heffron et al. (27) and two plasmids carrying a *Bam*HI linker in the cloned yeast DNA segment were selected. The site of insertion was confirmed by sequencing.

RESULTS AND DISCUSSION

Cloning of a DNA segment carrying the *PHO85* gene

Plasmid DNA prepared from the gene library was mixed with lithium acetate-treated YAT1175 cells and the mixture was spread on High-Pi medium supplemented with Casamino acid and tryptophan to select *Ura*⁺ transformants. Acid phosphatase activity of each colony was examined by the staining method. Most of *Ura*⁺ transformants were stained red due to the *pho85* mutation. Among approximately 5900 *Ura*⁺ transformants, we obtained 16 isolates which failed to be stained for acid phosphatase activity. Then, each transformant was cured of the plasmid by selecting *Ura*⁻ colonies and again acid phosphatase production in High-Pi medium supplemented with Casamino acid, tryptophan, and uracil was tested. Two transformants became constitutive with respect to the *Pho* phenotype when the plasmid was lost. Therefore, these two transformants contained plasmids that were candidates to carry the *PHO85* gene. Genomic DNA was isolated from each of these two transformants and used as donors for transformation of *E. coli* DHL from *Ap*^S to *Ap*^R. Plasmids thus obtained were designated pNF1 and pNF2. Introduction of either of these plasmids complemented the *pho85* defect of YAT1175. Comparison of the restriction maps of these two plasmids revealed a common region (Fig.1). In a strict sense, the DNA

TACAATCACCATACACTGAGACTCGTAAGCCCGTAGCTTCTTAATTGAAGATAACAAGGGAATCCAGTTTTACCATATAGCGTATAAATGCAGATAA 100
 ATGTTTAGTGGTATGTCGATCTGAGCATTGCGGCATCGAAAGATTAACTTCTATGTTTCCCTTAGGTCAAAATGGTATATCGCATATAAACCCTCTATT
 GGGATATATAGCGGGCAAACTGGGCAAACTTGAGCAATACCAATGCTTCTTCTTCCACAGTATGTAGTTTTCTAGTCAAGTATCATTGGAAAAGTAAAGA 200
 CCTATATATGCGCCGTTGACCCCTGTAACCTCGTTATGGTTFACAGAAGAAGAGTGCATACATCAAAAGATCAGTTCATAGTAAACCTTTCATTCT
 ACTAGAAATGATGAATACTAACATATATACGAGAAAAAATCAACCTCGA[●]GCTCTATGAACAGATTTAAGCAGTTAGAAAAGCTTGGCAATGGTACGTATG 300
 TGATCTTACTACTATGATGTATATATGCTCTTTTACTTGGAGCTCGAGA H N R F K Q L E K L G N G T Y
 CCACAGTGT[↓]CAAGGGAC[○]TGAACAAAAACACAGCCGATATATGTTGCCCTGAAAGAGGTA[■]AACTGGATTAGAGGAAAGTACACCCCTTACGGCCATCCG 400
 A T V Y K G L N K T T G V Y V A L K E V K L D S E E G T P S T A I R
 TGAGATCTCCCTAATGAAAGAATTGAAACATGAGAACCATTGTAGACTTATGACGTTATTACACAGAGAAACAGTTGACTTTGGTTTTGAAATTCATG 500
 E I S L M K E L K H E N I V R L Y D V I H T E N K L T L V F E F M
 GACAACGATTTAAAGAAATACATGATTC[▼]CCGCACCCTGGCAACACAC[○]CAAGAGGGCTAGAACTTAACTTGGTTAAATCTTCCAGTGGCAACTACTCG 600
 G D N D L K K Y M D S R T V A N T P R G L E L N L V K Y F Q W Q L L
 AAGGCTGGCC[○]TTTGGCATGAAAACAAGATTC[○]CCACCGTATTTAA[○]ACCCTAAAACCTATTAATCAACAAGAGAGGCCAGTTGAAATGGGTGATT 700
 Q G L A F C H E N K I L H R D L K P Q N L L I N K R G Q L K L G D F
 CGGTCTGGCCGCTGCTTCGTATTCGGTCAACACATTTCAAGCGAA[○]TGTAACGTTG[○]GGTACCGTCTCTGATGTGCTAATGGGTTCTAGGACG 800
 G L A R A F G I P V N T F S S E V V T L W Y R A P D V L M G S R T
 TACTCCACATCCATTGATATATGCTCGTGGGTGCATTCTTGGGAA[○]TGATAACGGGTAAGCCTTTGTTCTCGCCACCAACGACGAAGAACA[○]ACTGA 900
 Y S T S I D I W S C G C I L A E M I T G K P L F P G T N D E E Q L
 AATGATCTTCGACATCATGGGCACTCTAATGAGTCCCTATGGCCAGT[○]TAACAAGTTACCCAAATACAACCAAAATATCCAGCAACGACCAACG 1000
 K L I F D I M G T P N E S L W P S V T K L P K Y N P N I Q Q R P P R
 AGACCTACGTCAGTATTGCAACACACACCAAGAACCCTAGACGGG[○]ATCTCATGGATTCTTACACGGACTCTTGAACCTTAATCCGGATATGAGG 1100
 D L R Q V L Q P H T K E P L D G N L M D F L H G L L Q L N P D M R
 CTGAGCGCAAGCAGGCTCGATCAACCTTGGTTGCGAGTACTACC[○]ACCCTCATAACCGAGCCGTCAGCGAAAAACCGTAGCCATGTATATA 1200
 L S A K Q A L H H P W F A E Y Y H H A S ...GCCTCGGGCAGTCGCTTTTGGCATCGGTACATATAT
 TAATGATAATAATCACTATAATACACTACTATTACTTTAGTCTCATCTTACGACAGC
 ATTACTATATTAGTAGATAATTATGTGATGATAATGAAATCAGAGTAGAATGCTGTGCG

Fig. 3 Nucleotide sequence and deduced amino acid sequence of the PHO85 gene. The region where the nucleotide sequence of the both strands were determined is shown. \downarrow , AccI; \bullet , XhoI; \blacksquare , HindIII; \blacktriangledown , BglII; \circ , EcoRI; \square , KpnI.

segment which we cloned above contains a suppressor for the pho85 mutation. However, since it will later be proved that this gene is PHO85, we call the cloned gene PHO85.

Subclones and deletion derivatives were constructed and tested for their PHO85 activity by introducing each of them into YAT1175. Data shown in Fig.1 indicate that the complementing activity resides in the region encompassing the XhoI and EcoRI sites of the cloned DNA segment of pNF1. BamHI linkers were inserted at the indicated positions flanking the region of our interest without affecting the complementing activity.

DNA sequence of the 1258 bp fragment containing the PHO85 gene

Fig.2 shows the strategy for sequencing. The DNA fragments to be sequenced were inserted into an appropriate site of M13mp18 and M13mp19 and sequence determination was carried out by the dideoxy chain termination method (26). Fig.3 shows the nucleotide sequence of the 1258 bp DNA fragment containing the PHO85 activity. The largest open reading frame begins with ATG at position 255 and ends with TAA at position 1161. The deduced amino acid sequence is also shown in Fig.3. Calculated molecular weight of the putative protein is 34,674. Codon usage of the PHO85 gene is summarized in Table 1.

Table I. Codon usage of the PHO85 gene

Ala	GCT	4	Cys	TGT	1	His	CAT	3	Met	ATG	9	Thr	ACT	2
	C	6		C	2		C	7					C	4
	A	2											A	8
	G	1											G	5
Arg	CGT	5	Gln	CAA	8	Ile	ATT	6	Phe	TTT	6	Trp	TGG	5
	C	1		G	5		C	6		C	6			
	A	1					A	2						
	G	1												
	AGA	5												
	G	2												
Asn	AAT	5	Glu	GAA	11	Leu	TTA	6	Pro	CCT	6	Tyr	TAT	3
	C	13		G	7		G	11		C	3		C	8
							CTT	4		A	5			
							C	3		G	3			
							A	9						
							G	9						
Asp	GAT	9	Gly	GGT	7	Lys	AAA	12	Ser	TCT	2	Val	GTT	5
	C	6		C	4		G	10		C	5		C	2
				A	2					A	3		A	5
				G	5					G	1		G	3
										AGT	1			
										C	2			

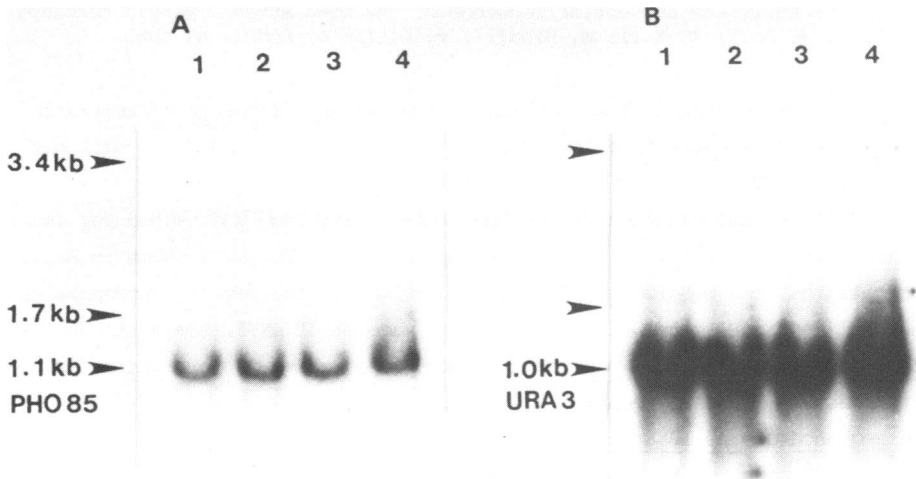


Fig. 4 Northern analysis of the PHO85 mRNA. RNA isolated from a set of the tetrad segregants of a cross between YAT954 and a pho85 mutant derived from YAT953 was applied to lane 1 through 4. Lanes 1 and 2, RNA from wild type strains grown in YEPD; lanes 3 and 4, RNA from pho85 mutants grown in YEPD. Filter hybridization was carried out with ³²P-labelled 1.8 kb HindIII fragment excised from pYU7 (A) or ³²P-labelled YIp5 (B) as probes. YIp5 was used to detect the URA3 mRNA. The conditions for hybridization were those described previously (9). Numbers at the sides are the size of the ribosomal RNAs used as the size markers (3.4 kb and 1.7 kb).

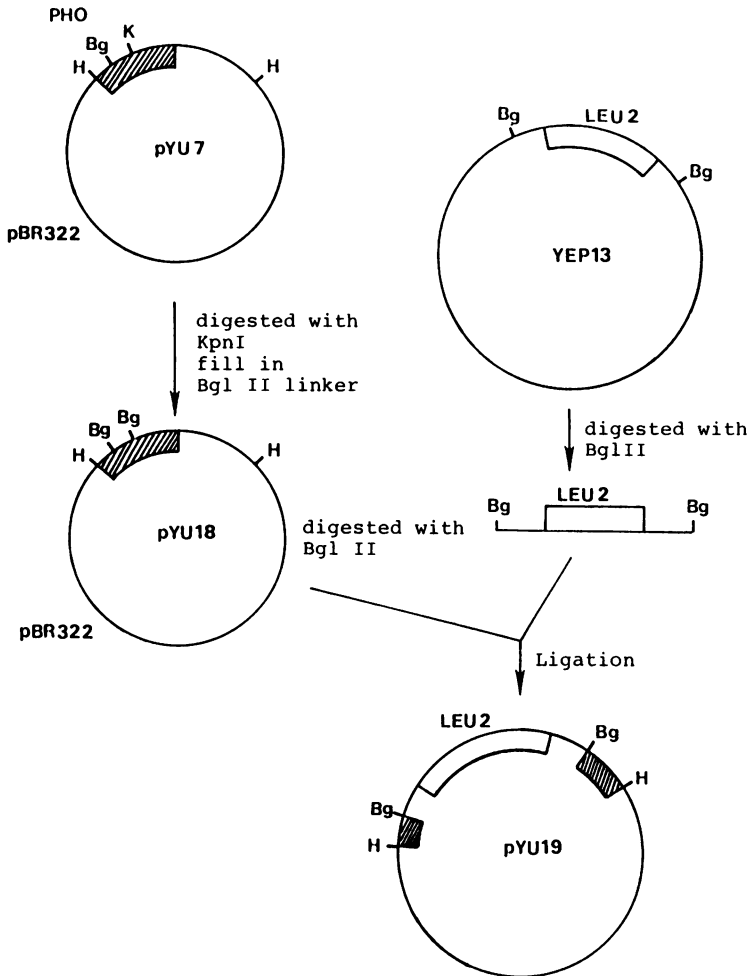


Fig. 5 Construction of plasmid pYU19 used for disruption of the PHO85 gene. Hatched boxes indicate a part of PHO85. Bg, BglIII. Other symbols are the same as those used in Fig.1.

Like other low expressing genes, codon usage of the PHO85 gene is poorly biased.

Northern analysis

Poly(A)⁺-RNA were prepared from wild type cells and pho85 cells grown in YEPD, separated on a denaturing agarose gel and blotted to a Biotyne membrane (Pall Ultrafine Filtration Corp. New York). Each slot contained approximately 15 µg RNA. Filter hybridization was carried out with the in-

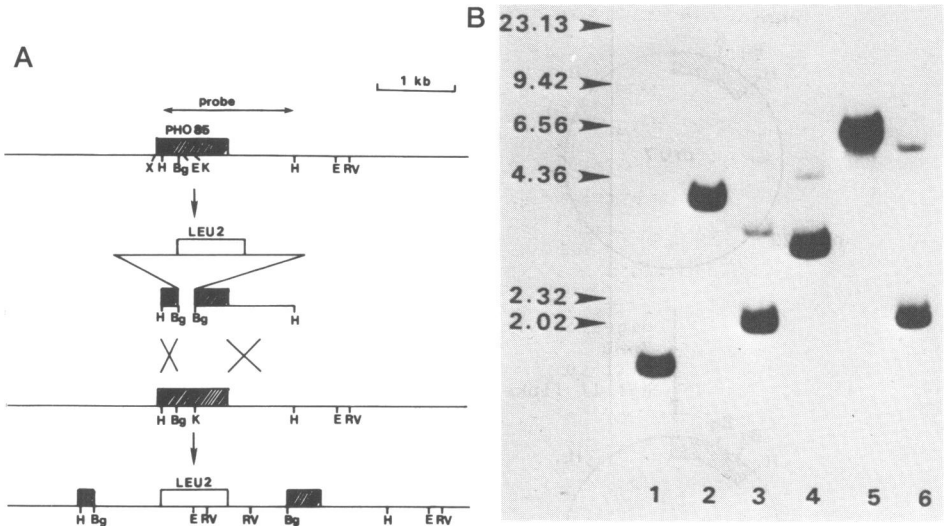


Fig. 6 Disruption of the PHO85 gene. (A) pYU19 DNA digested with HindIII was used as donor to transform YAT953 to Leu⁺. (B) DNA was extracted from one of the Leu⁺ transformants which produced acid phosphatase constitutively (YYU2) and digested with the indicated restriction endonucleases. DNA from DC5 was processed similarly. Southern analysis was carried out with the same probe used in Fig.4 at 65 °C in 3 x SSC (1 x SSC = 0.15M NaCl + 0.015M sodium citrate) containing 0.02% Ficoll 400, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 50 µg/ml sonicated and denatured calf thymus DNA, 0.1% sodium dodecyl sulfate, and ³²P-DNA. Lanes 1, 3, and 5 contained DNA from DC5 and lanes 2, 4, and 6 contained DNA from transformant, YYU2. Lanes 1 and 2, HindIII; lanes 3 and 4, EcoRI; lanes 5 and 6, EcoRV.

indicated probe labelled by *E. coli* DNA polymerase I and α -³²P-dCTP (28). As shown in Fig.4, one hybridization band was seen at the size of 1.1 kb in each lane. The figure also indicates that each lane contains comparable amounts of the 1.1 kb message.

Copy number of the PHO85 gene in the genome

Genomic DNA was isolated from DC5 cells and digested with the indicated restriction endonucleases and the resulting fragments were separated on an agarose gel. After transfer of fragments to a Biodyne membrane, filter hybridization was carried out using the 1.8 kb ³²P-labelled HindIII fragment of pYU7 as a probe. As expected from the restriction map shown in Fig.1, one 1.8 kb HindIII fragment (Fig.6B, lane 1) and one EcoRV band (8.1 kb, Fig.6B, lane 5) were seen. When DNA was digested with EcoRI (Fig.6B, lane 3), two bands, 2 kb and 3 kb, arose. This is also consistent with the previous map (Fig.1).

From these results, we concluded that the PHO85 gene is a single copy gene.

Disruption of PHO85

To prove that the gene we cloned is PHO85, the chromosomal counterpart of the largest open reading frame in the cloned DNA segment of pNF1 was disrupted and the phenotype of the disruptant was examined. For this purpose, a plasmid (pYU19) was constructed (Fig. 5). pYU7 contains the 1.8 kb HindIII fragment at the HindIII site of pBR322. pYU7 was digested with KpnI, rendered blunt with *E. coli* DNA polymerase I Klenow fragment, and then BglII linkers were attached to the ends. The plasmid thus obtained was designated pYU18. pYU19 was constructed by inserting the 2 kb DNA fragment containing the LEU2 gene into the BglII site of pYU18. The final result of the construction of pYU19 is the replacement of 1/3 of the coding region (409-767) of the PHO85 gene with the DNA fragment containing the LEU2 gene.

pYU19 was digested with HindIII and used as donor to transform YAT953 to Leu⁺. Many of the Leu⁺ transformants produced acid phosphatase constitutively. One clone (YYU2) was selected and the structure of the genomic DNA of YYU2 was analysed by Southern hybridization with the 1.8 kb ³²P-labelled HindIII yeast DNA fragment excised from pYU7 as a probe (Fig.6). One hybridization signal was seen in the lane containing the HindIII digest of the DC5 DNA sample at the size of 1.8 kb, which is the same size as that of the probe, whereas hybridization was seen at 3.8 kb in the HindIII digest of YYU2 DNA. EcoRI digestion gave rise to two bands in both DNAs, however, the sizes found in YYU2 differed from those found in DC5. EcoRV digestion of the wild type DNA gave rise to one 8.1 kb band, whereas the lane containing the EcoRV digest of YYU2 gave rise to two hybridization signals; one is at 2.5 kb and another at 6.6 kb. This hybridization pattern can be explained by the predicted replacement of the HindIII fragment of pYU19 for the chromosomal counterpart.

The constitutive transformant, YYU2, was crossed with the pho3 strain, YAT954. The resulting diploid showed a wild type phenotype. A diploid prepared by crossing YYU2 with a standard pho85 strain showed a constitutive phenotype; the mutation induced by the disruption did not complement the pho85 mutation. These results proved that the cloned gene is the PHO85 gene. When the heterozygous diploids constructed above were sporulated and dissected Pho phenotype segregated 2:2 and the two constitutive clones were always slow growers.

To understand the regulatory mechanisms working in the PHO system, it is necessary to characterize the gene product of each of the regulatory genes at the molecular level. Cloned regulatory genes make such approach possible.

Data presented in this study, along with data obtained previously, will help test the validity of the model at the molecular level.

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