Primary Structure and Disruption of the Phosphatidylinositol Synthase Gene of Saccharomyces cerevisiae*

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The wild-type yeast nuclear gene, PIS, encodes phosphatidylinositol synthase (CDP-diacylglycerol-inositol 3-phosphatidyltransferase, EC 2.7.8.11) (Nikawa, J., and Yamashita, S. (1984) Eur. J. Biochem. 143, 251-256). We now report the sequence of the cloned 2,129-base pair DNA and the location of the PIS coding region within the sequence. The PIS coding frame is capable of encoding 220 amino acid residues with a calculated molecular weight of 24,823. On Northern blot analysis, an RNA species that hybridized with the coding region was detected in the total poly(A)+ RNA of the wild-type yeast.

The primary translation product contains a region showing local sequence homology with yeast phosphatidylserine synthase (EC 2.7.8.9) and Escherichia coli 3-phosphatidy-1'-glycerol-3'-phosphatase synthase (EC 2.7.8.5), suggesting that these three enzymes are evolutionarily related.

The PIS gene was disrupted in vitro through insertion of the yeast HIS3 gene into the coding region. A heterozygous diploid, PIS/pis::HIS3, constructed from a PIS/PIS his3/his3 diploid by replacing one of the wild-type PIS genes with the disrupted PIS gene, showed no segregation of viable His+ spores on tetrad analysis, indicating that disruption of the PIS gene is lethal. The nonviable spores were in an arrested state with a characteristic terminal phenotype, suggesting that the function of the PIS gene is essential for progression of the yeast cell cycle.

Phosphatidylinositol is synthesized from CDP-diacylglycerol and myo-inositol by phosphatidylinositol synthase (CDP-diacylglycerol-inositol 3-phosphatidyltransferase, EC 2.7.8.11) and is converted by sequential phosphorylation into phosphatidylinositol 4,5-bisphosphate via phosphatidylinositol 4-phosphate. Phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4,5-bisphosphate are also present in this organism (8) and undergo rapid turnover (9). There is considerable evidence that myo-inositol-containing phospholipids play important roles in yeast cells like in mammalian cells. When myo-inositol-requiring mutants of S. cerevisiae are deprived of myo-inositol, biochemical and morphological changes occur and ultimately result in a loss of cell viability (10-13). This loss of viability, called inositol-less death, is rapid and is considered to arise from impairment of membrane functions as a result of the inositol phospholipid deficiency.

In an attempt to define the functions of inositol phospholipids in the yeast, we isolated a mutant containing an altered phosphatidylinositol synthase (14). The mutant required a high concentration of myo-inositol for phosphatidylinositol synthesis because the mutant enzyme had an apparent Km for myo-inositol of over 200 times higher than that of the wild-type enzyme. Under the conditions of myo-inositol limitation, cells ceased phosphatidylinositol synthesis and stopped growing. Using this mutant we cloned the structural gene that encodes phosphatidylinositol synthase (PIS) from a wild-type yeast DNA library by means of genetic complementation (15). The present study was undertaken to characterize the cloned PIS gene and to investigate the physiological effects of disruption of the PIS locus of the yeast genome. The gene was found to be capable of directing the synthesis of a protein composed of 220 amino acid residues. An mRNA with a nucleotide length of approximately 1.2 kb was detected. The deduced amino acid sequence of the phosphatidylinositol synthase showed local homologies with those of phosphatidylerine synthase and phosphatidyglycerophosphate synthase. On disruption of the genomic PIS locus by means of gene transplantation it was established that the PIS gene function is essential for yeast growth. Cells lacking this function cease division and exhibit a characteristic terminal phenotype.

EXPERIMENTAL PROCEDURES

Strains and Media—S. cerevisiae strain D378-2A (a pis leu2 his4), carrying a mutation in the phosphatidylinositol synthase structural gene (15), was used in the cloning and subcloning experiments. Strain D111 (a/a PIS/PIS leu2/leu2 his3/his3 trpl1/trpl1) was constructed by a standard genetic method and used as the wild-type diploid strain in the gene disruption study. Yeast cells were grown aerobically with

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The abbreviations used are: kb, kilobases; kbp, kilobase pairs; bp, base pairs.
shaking at 30 °C. The compositions of minimum medium M2 and complex medium WaD were as described previously (16). L-Leucine, L-histidine, and L-tryptophan were added to the culture media at concentrations of 20 μg/ml. Escherichia coli K12 strain ML4001 (17) was obtained from Dr. Inoue (Gunma University) and used for the maintenance and amplification of all plasmids used in this study. E. coli cells were grown in Luria broth (18) or χ broth (15) at 37 °C. Ampicillin was used at a final concentration of 50 μg/ml.

Plasmids—Plasmid pPI514 (15) was used as a source of PIS DNA for the sequencing study. Plasmid pPI535, a deletion derivative of pPI514 lacking the 1.0-kbp SalI/XhoI fragment, was constructed by ligating the BamHI/XhoI fragment of pPI514 with the YEp13 vector which had been digested completely with BamHI and then partially with SalI.

Transformation of the Yeast and E. coli—Unless otherwise specified, the procedure of Beggs (19) was used for yeast transformation. The transformation of E. coli was performed as described (18) with a slight modification (15). Unless otherwise stated, recombinant DNA experiments were carried out according to Maniatis et al. (20).

DNA Sequencing—Restriction fragments were cloned into M13 derivatives mp10 and mp11 by the method of Messing et al. (21). Sequence determinations were performed by the dyeoxy chain-terminating method of Sanger et al. (22) using an M13 pentadecamer primer (Amersham Corp.). The nucleotide sequence was analyzed using at least two overlapping clones.

Northern Blot Hybridization—Poly(A)− RNA was prepared from a logarithmic culture of wild-type strain X2180-1B (a SUC2 mal gal2 CUT1) (Sacramento Genetic Stock Center, University of California) as described by Kataoka et al. (23) and then electrophoresed in an agarose gel containing formaldehyde (20). Blotting onto a Biodyne A membrane (Nihon Pole) and hybridization were carried out according to the manufacturer's recommendations. Nick-translation of the 1.6-kbp HindIII fragment of the pPI514 insert was performed using an Amersham Corp. nick-translation kit and then it was used as the probe.

Gene Disruption—The 996-bp EcoR1 fragment of pPI535 was transferred to pUC19 to yield pPI536, pPI536 was cleaved at the Neo site within the PIS coding frame and ligated to a 1.7-kbp BamHI fragment carrying the yeast HIS3 gene (24) through the consecutive use of the Klenow fragment and T4 ligase. The linear 2.7-kbp EcoR1 fragment of this plasmid (pPI537) was used for transformation of diploid yeast strain DJ11 according to the lithium acetate method of Ito et al. (25). His+ colonies were selected and gene transplacement was confirmed by Southern blot analysis (26).

Enzyme Assay—Phosphatidylinositol synthase activity was assayed using the membrane fraction as described previously (14).

RESULTS AND DISCUSSION

Nucleotide Sequence of the PIS Gene—PIS, the structural gene that encodes phosphatidylinositol synthase, was originally identified as a 4.8-kbp insert cloned on plasmid YEp13 (27) through genetic complementation in a πis mutant D278-2A (a πis leu2 his4) (15). Subcloning experiments showed that the PIS gene is located in the 2.2-kbp insert of plasmid pPI514 (Fig. 1) (15). Therefore, we determined the nucleotide sequence of the insert of plasmid pPI514 by the dyeoxy method (22) using derivatives of the M13 phage (21). The restriction map and the sequencing strategy are presented in Fig. 2. The complete nucleotide sequence is shown in Fig. 3. Within this sequence there are two long open reading frames. One encompasses nucleotide positions 54 through 982 that is capable of encoding 143 amino acid residues, and the other is composed of 220 codons starting from nucleotide 918 and ending at nucleotide 1577 according to the base number coordinates given in Fig. 3.

To determine which open reading frame encodes phosphatidylinositol synthase, we constructed pPI535, which is deficient in the first open reading frame, by removing the 1.0-kbp SalI/XhoI fragment from the pPI514 insert (Fig. 1), and then introduced it into yeast χis strain D278-2A. The plasmid directed the synthesis of phosphatidylinositol synthase in the transformant with 11-fold overproduction of the enzyme (20.2 nmol/min/mg protein), indicating that the nucleotide sequence between positions 1 and 792 is dispensable. Thus, the open reading frame of the PIS gene can be assigned to the region encompassing nucleotide positions 918-1577. The yeast intron splice signal, TACTAAC (28), was not found. The open reading frame is preceded by a TATATAA sequence (29) and a TATA transcriptional start signal of eukaryotic genes (29). At position +4 relative to the A of the ATG codon, there is a pyrimidine base, T, as found by Dobson et al. (31) in yeast genes. The calculated molecular weight of the primary translation product of the PIS gene is 24,823. There are two potential sites for N-linked glycosylation, Asn-Ser-Thr and Asn-Lys-Thr (32), with Asn at positions 220 codons starting from nucleotide 918 and ending at nucleotide 1577 according to the base number coordinates given in Fig. 3.

The coding region of the PIS gene is terminated by a single termination codon, the histone H2B1 (33), TRP5 (34), and phosphatidylycerine synthase2 genes are followed by two stop codons. An AATAAA polyadenylation signal (35,36) can be seen 156 nucleotides downstream from the coding frame. We also found the TAG...TATGT...TTT sequence proposed to be a yeast transcriptional termination signal by Zaret and Sherman (37) between nucleotides 1639 and 1676 of the 3′-flanking region.

Detection of the PIS mRNA—To determine whether or not the putative open reading frame was actually transcribed in

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yeast cells, we performed Northern blot analysis of wild-type yeast RNA using a DNA sequence containing the PIS coding region as the probe. Poly(A)* RNA was prepared from wild-type yeast strain X2180-1B, electrophoresed in a formaldehyde-containing gel, and then transferred to a Biodyne A membrane as described under “Experimental Procedures.” The membrane was hybridized with the nick-translated 1.6-kbp HindIII fragment of the pP1514 insert. Yeast 25 S (3.36 kb) and 18 S (1.71 kb) ribosomal RNA were used as size markers.

**Fig. 3.** The nucleotide sequences of the PIS gene and the surrounding regions. The coding frame for phosphatidylinositol synthase is translated. Dashed lines indicate putative N-glycosylation sites. The location of a putative transcription start signal in the 5′-flanking region is underlined. The nucleotide sequence with a wavy underscore indicates the poly(A)* tail addition signal. The putative yeast transcription termination signal is dotted.

![Phosphatidylinositol Synthase Gene](image)

**Table 1**

| Codon usage in the PIS gene |
|-----------------------------|
| Asterisks indicate termination codons. |
|UUU-Phe | 5 | UCU-Ser | 3 | UAU-Tyr | 6 | UGU-Cys | 4 |
|UUC-Phe | 8 | UCC-Ser | 3 | UAC-Tyr | 7 | UGG-Cys | 1 |
|UUA-Leu | 4 | UCA-Ser | 3 | UAA*** | 0 | UGA*** | 1 |
|UUG-Leu | 8 | UCG-Ser | 1 | UAG*** | 1 | UGG-Trp | 4 |
|CUU-Leu | 2 | CUC-Pro | 2 | CAU-His | 3 | CGU-Arg | 0 |
|CUC-Leu | 0 | CCC-Pro | 2 | CAC-His | 3 | CGC-Arg | 0 |
|CAA-Leu | 4 | CCA-Pro | 1 | CGA-Gln | 2 | CGG-Arg | 0 |
|CAG-Leu | 7 | CGG-Pro | 0 | CAG-Gln | 0 | CCG-Arg | 0 |
|AUG-Arg | 13 | UAA*** | 6 | AAA-Asn | 5 | AGA-Ser | 6 |

**Codon Usage**—A distinct bias has been observed in the frequency at which a particular degenerate codon is used to code for a particular amino acid in both prokaryotic and eukaryotic genes. There is a good correlation between the degree of the bias in codon selection and the extent of expression of a gene. A high level of gene expression was found to be well associated with a high codon bias level (28). In the PIS gene, as in other yeast genes, there is a bias toward the use of codons with pyrimidines at the third positions for Ile, Thr, and Gly (Table I). However, little bias was seen in the use of codons with purines at the third positions for Tyr, His, Asn, and Glu. Bennetzen and Hall (38) defined the codon bias index as a measure of the fraction of codon choices which is biased to the 22 triplets preferably used in yeast genes. The calculated codon bias index of the PIS gene is low, 0.21. Therefore, it is unlikely that the PIS gene is a highly expressed gene. This view is consistent with the low enzyme activities detected in yeast cells.
FIG. 5. Hydrophathy profile of the PIS gene product. The ordinate shows the average hydrophathy index of a stretch of 9 residues and the abscissa, the residue number at the center of the stretch. The base line represents the average hydrophathy value for several soluble proteins determined according to Kyte and Doolittle (39).

Hydrophobic Profile—Fig. 5 shows the hydrophathy profile of the PIS gene product displayed by the method of Kyte and Doolittle (39). Phosphatidylinositol synthase is localized in microsomes and outer mitochondrial membranes (40). The requirement of a detergent for its solubilization (41) indicates that the enzyme protein constitutes an integral part of the membrane. The grand average hydrophathy of the enzyme is 0.07. This value lies well above the mean of the values for soluble proteins (−0.4) (39). The polypeptide chain contains several hydrophobic stretches within its sequence. Among them, the stretches encompassing residues 88–109 (average hydrophathy index, 1.5) and 149–169 (average hydrophathy index, 1.5) are sufficiently extended and hydrophobic to be membrane-associated.

Sequence Homologies between Phosphatidylinositol Synthase and Other CDP-Diacylglycerol-utilizing Enzymes—Elucidation of the nucleotide sequence of the PIS gene allowed comparison of the amino acid sequence of phosphatidylinositol synthase with those of other functionally related enzymes. A search through the sequence data for enzymes that utilize CDP-diacylglycerol as a substrate revealed regions showing high degrees of local homology with phosphatidylinositol synthase (E. coli) (42). As can be seen in Fig. 6, alignment of the partial sequences of the three enzymes does not require any insertions or deletions. Out of the 48 amino acid residues compared, 10 are identical and 4 are conservative substitutions in the three sequences. Asp residues are invariably conserved. Comparison of phosphatidylinositol synthase with phosphatidylylglycerophosphate synthase shows the presence of 18 identical residues and 5 conservative substitutions in the region compared. Comparison with phosphatidylerine synthase indicates that 13 residues are identical and 3 are conservative substitutions. The statistical significance of the homology between the compared sequences was checked using the computer program of Kanehisa (43). The significances expressed in standard deviation units were 1.71 (between phosphatidylerine synthase and phosphatidylinositol synthase), 9.26 (between phosphatidylinositol synthase and phosphatidylylglycerophosphate synthase), and 3.55 (between phosphatidylerine synthase and phosphatidylylglycerophosphate synthase), indicating that the homologies of the E. coli phosphatidylylglycerophosphate synthase with the yeast phosphatidylinositol synthase and phosphatidylerine synthase are highly significant. The high degree of conservation of the amino acid sequence in these functionally related enzymes probably reflects their functional constraints and suggests that they share a common evolutionary ancestor.

An additional finding of interest in this context is that we failed to find significant homology with the E. coli CDP-diacylglycerol hydrolase sequence (44). This enzyme catalyzes the hydrolysis of CDP-diacylglycerol to phosphatidic acid and CMP. Alternatively, it also catalyzes the transfer of the CMP moiety of CDP-diacylglycerol to other acceptors, suggesting the occurrence of an enzyme CMP complex (45). In contrast, phosphatidylinositol synthase catalyzes the transfer of the phosphatidate moiety of CDP-diacylglycerol to myo-inositol to give phosphatidylinositol. A recent study using highly purified yeast phosphatidylinositol synthase reconstituted into liposomes indicated that the enzyme neither exhibits a ping-pong reaction mechanism nor catalyzes the hydrolysis of CDP-diacylglycerol (46). Although both phosphatidylinositol synthase and CDP-diacylglycerol hydrolase use CDP-diacylglycerol as a common substrate, they exhibit distinct reaction mechanisms and may be evolutionarily less related.

Disruption of the Genomic PIS Locus—In previous studies (14), we demonstrated that an alteration in the phosphatidylinositol synthase enzyme with the elevation of the apparent \( K_m \) for myo-inositol leads to the requirement of myo-inositol by cells for both phosphatidylinositol synthesis and growth (14). This observation led us to suppose that phosphatidylinositol synthase is indispensable for yeast growth. The complete elucidation of the primary sequence of the phosphatidylinositol synthase gene allowed us to disrupt the PIS locus of the yeast genome and to examine its effect on the growth of yeast cells. The in vitro disruption of the PIS gene through insertion of the yeast \( HIS3 \) gene is shown in Fig. 7. The 996-bp EcoRI fragment of the pPI535 insert (Fig. 1) containing the entire PIS coding sequence except for the eight 5′-end nucleotides was cloned into the pUC19 vector to yield pPI536. A 1.7-kbp BamHI fragment carrying the yeast \( HIS3 \) gene (24) was ligated into the NcoI site of pPI536 inside the PIS coding sequence. The 2.7-kbp EcoRI fragment of the resulting plasmid, pPI537, containing the disrupted \( PIS \) was introduced into wild-type diploid DJ11 (\( a/aPIS/PIS\) leu2/leu2 his3/his3 trp1/trp1), followed by selection for His+ colonies. Chromosomal integration of the disrupted gene was confirmed by the stable His+ phenotype after culturing in a nonselective medium (WaD).

To confirm that gene transplacement had occurred at the \( PIS \) locus in the genome, Southern blot analysis of the genomic DNA was carried out. Total yeast DNA from the parental strain, DJ11, and a transformant, DJ1101, was di-
FIG. 7. \textit{In vitro} disruption of the \textit{PIS} gene by insertion of the yeast \textit{HIS3} gene. The 996-bp EcoRI fragment of pPI535 carrying most of the \textit{PIS} coding region was cloned into pUC19 (pPI536). The \textit{PIS} gene was disrupted by inserting the 1.7-kbp yeast \textit{HIS3} gene (indicated by the \textit{solid} region) into the NcoI site within the \textit{PIS} coding region as described under “Experimental Procedures.” The resulting plasmid, pPI537, was cleaved with EcoRI and the linear 2.7-kbp fragment obtained was used to transform diploid strain DJ11 (a/α \textit{PIS}/\textit{PIS} leu2/leu2 his3/his3 trpl/trp1). His+ transformants were selected as described under “Experimental Procedures.” Single lines indicate the pUC19 vector sequence. Boxes indicate the yeast sequence. The open box represents the \textit{PIS} coding frame, and the hatched box, the 3′-flanking region. The abbreviations used for restriction enzyme sites are: \textit{E}, EcoRI; \textit{H}, HindIII; \textit{N}, NcoI; \textit{P}, PstI; \textit{J}, junction of the NcoI and BamHI sites.

FIG. 8. Southern blot analysis of the disrupted genomic \textit{PIS} locus. Total yeast genomic DNA from wild-type parental diploid DJ11 and the His+ transformant (DJ1101) and its meiotic segregants was digested with EcoRI, electrophoresed on a 0.7% agarose gel, transferred to a nitrocellulose sheet, and then hybridized with the radioactively labeled 996-bp EcoRI fragment of pPI535 containing most of the \textit{PIS} coding region. Lane 1, wild-type diploid DJ11; lane 2, heterozygous diploid carrying one copy each of the wild-type and \textit{HIS3}-disrupted genes; lanes 3–6, viable spores obtained on tetrad dissection. A HindIII digest of λ phage DNA was used as size markers.

FIG. 9. Tetrads dissection of meiotic segregants of heterozygous diploid DJ1101 (a/α \textit{PIS}/\textit{PIS}:\textit{HIS3} leu2/leu2 his3/his3 trp1/trp1). The diploid was induced to sporulate on acetate medium, and then tetrads were dissected by micromanipulation. Five tetrads which grew on a W+D plate at 30°C for 4 days are shown. Only two spores for each ascus were viable. Tetrads derived from the same asci are aligned in horizontal rows.

FIG. 10. Morphology of the arrested cells. Nonviable cells derived from diploid DJ1101 (a/α \textit{PIS}/\textit{PIS}:\textit{HIS3} leu2/leu2 his3/his3 trp1/trp1) were examined under a microscope. \textit{A}, an arrested cell with one large bud; \textit{B}, an arrested cell with two large buds.

DNA within the chromosomal \textit{PIS} DNA. Comparison of the hybridization intensities of the intact and disrupted \textit{PIS} bands suggested that a single copy of \textit{PIS} is present in the yeast genome. Replacement of the genomic \textit{PIS} locus with the disrupted \textit{PIS} gene was further confirmed using the HindIII digest of the genomic DNA from the transformant (data not shown). The present results show that one of the two \textit{PIS} genes of the transformant is disrupted on the insertion of the 1.7-kbp \textit{HIS3} gene.

The heterozygous diploid, DJ1101, thus obtained (a/α \textit{PIS}/\textit{PIS}:\textit{HIS3} leu2/leu2 his3/his3 trp1/trp1) was induced to undergo meiosis on acetate medium. Tetrads dissected revealed that the heterozygous diploid yielded only two viable spores (Fig. 9). All of the viable spores are His+. Southern blot analysis of DNA isolated from several viable spores indicated that they contain the wild-type \textit{PIS} gene (Fig. 8). These results show that disruption of the \textit{PIS} locus is lethal for yeast cells. Microscopic examination indicated that the spores which contain the \textit{HIS3}-disrupted \textit{PIS} gene germinate, undergo budding, and then become arrested. The arrested cells exhibited a terminal phenotype with one or two large buds. Of the nine cells examined, four were arrested with one large bud (Fig. 10A), and the other five were arrested with two large buds (Fig. 10B). These results indicate that the function of the \textit{PIS} gene is required for progression of the...
cell division cycle. It is suggested that the PIS gene can be classified as one of the CDC genes (47).

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