The *PPS1* Gene of *Saccharomyces cerevisiae* Codes for a Dual Specificity Protein Phosphatase with a Role in the DNA Synthesis Phase of the Cell Cycle

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We report the identification of the *PPS1* gene of *Saccharomyces cerevisiae*. The deduced amino acid sequence of *PPS1p* shows similarity with protein-tyrosine phosphatases (PTPases) and is most closely related to a subfamily of PTPases that are capable of dephosphorylating phosphoseryl and phosphothreonyl residues as well as phosphotyrosyl residues. Analysis of the predicted amino acid sequence suggests that the protein consists of an active phosphatase domain, an inactive phosphatase-like domain, and an NH2-terminal extension. Mutation of the catalytic cysteinyl residue in the active phosphatase domain reduced the *in vitro* activity of the mutant protein to less than 0.5% of wild type activity, while mutation of the corresponding cysteinyl residue of the inactive phosphatase-like domain had no effect on *in vitro* activity. The *PPS1* protein was expressed in *Escherichia coli*, and the protein was shown to catalyze the hydrolysis of *p*-nitrophenyl phosphate, dephosphorylate phosphotyrosyl, and phosphothreonyl residues in synthetic diphosphorylated peptides and to inactivate the human ERK1 protein. *PPS1* transcript abundance is coregulated with that of the divergently transcribed *DBP3* gene, which codes for a subunit of DNA polymerase II, with both transcripts showing peak abundance in S phase. *pps1Δ* mutant strains did not differ from *PPS1* strains under any of the conditions tested, but overexpression of the *PPS1* protein in *S. cerevisiae* led to synchronous growth arrest and to aberrant DNA synthesis. A screen for suppressors of this growth arrest identified the *RAS2* gene as a multicopy suppressor of the *PPS1* overexpression arrest. The arrest was not suppressed by the presence of multicopy *RAS1, TPK2*, or *TPK3* genes or by the presence of 5 mM cAMP in the growth medium, suggesting that *PPS1* functions in a pathway involving *RAS2*, but not TPK kinases or adenylate cyclase.

Protein-tyrosine phosphorylation is a key regulator of a number of cellular processes. Substrate proteins may be phosphorylated by protein kinases and dephosphorylated by protein phosphatases. These reactions are regulated in response to environmental conditions, with the phosphorylation state of substrate proteins serving as an indicator of the appropriate response. The level of phosphorylation of target proteins, and thus the level of response, depends on three factors: the rate of enzymatic phosphorylation, the intrinsic stability of the phosphorylated amino acid, and the rate of enzymatic dephosphorylation. Since phosphotyrosyl residues are quite stable, the level of target phosphorylation depends on the interplay between kinases and phosphatases. In yeast, the response to mating pheromones (1) and medium osmolarity (2) as well as cell cycle events including control of initiation of mitosis (3) have been shown to involve phosphorylation and dephosphorylation of critical protein tyrosyl residues.

In the mating reaction, peptide pheromones are secreted by haploid cells according to their mating type (a or α). These pheromones bind to specific receptors on cells of the opposite mating type and initiate a signaling pathway that leads to arrest of the cell cycle in G1 and to altered gene expression and cell morphology (4). The kinases FUS3p and KSS1p, members of the MAP kinase family, are activated as part of this cascade.

Another MAP kinase family member, HOG1p, is involved in the regulation of the *S. cerevisiae* response to changes in medium osmolarity (5, 6). The activity of CDC28p cyclin-dependent kinase homologs is also modulated by tyrosine phosphorylation (for a review, see Ref. 7). In *S. pombe*, the CDC28 homolog CDC2 is inactivated by a specific tyrosine phosphorylation catalyzed by the Wee1 kinase. Dephosphorylation of this tyrosyl residue by the phosphatase CDC25 results in active CDC2 and allows entry into M phase (8, 9).

Five protein-tyrosine phosphatases have been identified in *S. cerevisiae*. The PTP1 (10) and PTP2 (11) genes were identified using degenerate PCR primers based on conserved regions of protein-tyrosine phosphatases (PTPases). Conserved residues at the PTPase active site include the sequence HCVGXX/S/T, the “signature sequence” of PTPase active sites. The cysteine in this sequence is invariant in PTPases, and mutation of this residue to serine or alanine abolishes PTPase activity (12). These active site residues are contained in a larger sequence of approximately 250 amino acids that define the PTPase domain. Selection for synthetic lethal mutations in *PTP2* mutant strains demonstrated that *PTP2* has functional overlap with the protein serine/threonine phosphatase *PTC1* (13) and that these phosphatases are important for the inactivation of the HOG1 kinase (2). The observation that either a protein-tyrosine phosphatase or a serine/threonine phosphatase is sufficient to regulate a MAP kinase kinase-MAP kinase pair suggests that other PTPases may cooperate with protein serine/threonine phosphatases in this way. A third PTPase gene, *YVH1*, which also shares the PTPase signature

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The abbreviations used are: MAP, mitogen-activated protein; PTPase, protein-tyrosine phosphatase; bp, base pair(s); kb, kilobase pair(s); GST, glutathione S-transferase; PBS, phosphate-buffered saline; p-NPP, p-nitrophenyl phosphate.
sequence, was identified by analysis of a partially sequenced open reading frame. *YHV1* mRNA levels are increased when cells are grown under nitrogen-limiting conditions, and genetic deletion of *YHV1* results in a decrease in growth rate (14). Another PTPase, the CDC14 gene product, has been shown to be required for viability. CDC14 was initially characterized as a temperature-sensitive mutation (cdc14) that caused cells to arrest at the late nuclear division stage of the cell cycle when incubated at the nonpermissive temperature (15). Cloning and analysis of the CDC14 sequence showed that CDC14p contains the PTPase signature sequence (16). Recently, a fifth PTPase, MSG5, was isolated as a suppressor of a conditional lethal mutation in the GPA1 gene. MSG5 transcription is induced upon exposure to mating pheromone, and deletion of this gene reduces the ability of cells to adapt to the presence of pheromone. Together, these results suggest a role for MSG5 in recovery from G1 arrest following exposure to mating pheromone and suggest that the kinase FUS3p, which must be dephosphorylated for recovery from G1 arrest, is a substrate for MSG5p (17).

Continuing progress in determining the sequence of the *S. cerevisiae* genome allows us to identify open reading frames that are likely to encode PTPase activity. In this work, we report the isolation and initial characterization of a sixth *S. cerevisiae* protein-tyrosine phosphatase. The PPS1 gene was identified in a search of DNA sequence data bases with the PTPase signature sequence. Analysis of the deduced amino acid sequence of PPS1p suggests that the protein contains an active phosphatase domain as well as an inactive phosphatase-like domain. Activity assays of wild type protein and site-directed catalytic mutant proteins confirm that the phosphatase activity of the wild type protein resides in the C-terminal phosphatase domain. We have shown that PPS1p is capable of dephosphorylating phosphotyrosyl and phosphothreonyl residues in synthetic peptides. Although PPS1 is not an essential gene, we have shown that overexpression of the PPS1 protein leads to synchronous growth arrest and to aberrant DNA synthesis. This overexpression arrest is suppressed by the presence of the *RAS2* gene on a multicopy plasmid. PPS1 transcript abundance fluctuates during the cell cycle, with peak abundance in S phase. We have named the novel phosphatase PPS1 for protein phosphatase S phase.

**MATERIALS AND METHODS**

**Yeast Strains and Growth Conditions**—Yeast strains (Table I) were grown in rich medium (YPEP; 1% yeast extract, 2% bacto-peptone, with 2% dextrose as carbon source) or synthetic medium (SC; 0.67% yeast nitrogen base without amino acids, supplemented with nutrients as described (18), with 2% dextrose, galactose, acetate, or glycerol as carbon source.) All cultures were grown aerobically in rotary shakers at 30 °C. Cell growth was monitored spectrophotometrically at 600 nm. For α-factor synchronization experiments, cells were grown in SC, 2% dextrose to an OD600 of 0.5. *S. cerevisiae* α-factor (Sigma) was added to a final concentration of 5 μg/ml, and the culture was incubated at 30 °C with shaking for 2 h. Cell morphology was monitored microscopically to confirm growth arrest. Cells were collected by centrifugation at room temperature and resuspended in prewarmed SC, 2% dextrose medium. 

**Bacterial Strains and Growth Conditions**—Escherichia coli strain DH5α was used for routine transformation and plasmid DNA preparation. Strain BL21/DE3 (Novagen) was used as a host strain for protein expression. *E. coli* cultures were grown aerobically at 37 °C in rich (2% BYE, 1.6% Bacto-peptone, 1% yeast extract, 0.5% NaCl) medium. Where plasmids carrying the ampicillin resistance gene were present, media contained ampicillin at a final concentration of 100 μg/ml.

**Cloning and Plasmid Construction**—Primers containing PPS1 sequence (5′-CCGACAACCTCCATTCCAC-3′ and 5′-AGGATTGTTGACATGCATCTCAGG-3′) for the upstream and downstream primers, respectively, were used to amplify a 678-bp product from *S. cerevisiae* chromosomal DNA. The polymerase chain reaction was carried out with denaturant at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. This product was cleaved with BglII and HindIII and ligated into the BamHI and HindIII sites of plasmid pGEM3Z (Promega) to produce plasmid pBE20. The PPS1 sequence from pBE20 was then used as a probe to screen a *S. cerevisiae* chromosomal library (CENA library; ATCC). Bacterial colonies containing library plasmids were grown on selective medium and transferred to nitrocellulose filters. Colonies with plasmids containing PPS1 sequence were detected by colony hybridization. The DNA probe was labeled with [α-32P]dCTP (NEN) in a random primed labeling reaction (Boehringer Mannheim). Hybridization was carried out in 50% formamide, 5× Denhardt’s solution, 5× SSPE, 0.1% SDS, and 100 μg/ml salmon sperm DNA at 42 °C for 18 h. Filters were washed in 1× SSC, 0.1% SDS at 85 °C. 

**Plasmid Preparation**—The resulting plasmid DNA was isolated from these colonies, and a 3561-base pair EcoRI-Nhel fragment containing the PPS1 open reading frame was ligated into the EcoRI and Nhel sites of plasmid pUC119 to produce plasmid pBE22. A plasmid carrying a disrupted pps1A gene (Fig. 1) was constructed by cutting pBE22 with BglII. This digestion removes PPS1 sequence from nucleotides 718–2192, relative to the presumptive ATG initiator codon, and results in the deletion of PTPase active site signature from the PPS1 gene. In place of this sequence, a 3.8-kb insert derived from a BglII BamHI PvuII digest of plasmid pNY51 (19) was inserted, producing plasmid pBE24. pBE24 contains a URA3 selectable marker, flanked by PPS1 and adjacent sequence, and was used in the gene disruption procedure.

To express the PPS1 protein in *E. coli*, plasmid pBE22 was cut with NcoI and SalI. The resulting 3313-bp fragment containing the PPS1 open reading frame was inserted into the NcoI and SalI sites of plasmid pGEX-KG (20). The resulting plasmid, pBE25, contains the PPS1 coding sequence fused in frame to glutathione S-transferase (GST), with the twoproteinase sites separated by a thrombin cleavage site and a polyglycine kinker. Cleavage of the fusion protein yields glutathione S-transferase and PPS1p products. The PPS1 product of this reaction contains an additional 15 amino acids specified by codons in the linker region NH2-terminal to the initiator methionine of the PPS1 reading frame.

A vector capable of overexpressing PPS1p in *S. cerevisiae* was constructed using an EcoRI XhoI fragment of pBE25. This fragment, containing the PPS1 coding sequence, was gel-isolated and ligated into the EcoRI XhoI sites of plasmid pYES2AT, a derivative of pYES2 (Invitrogen). The resulting vector, plasmid pBE33, contains the URA3 and AmpR selectable markers for use in *S. cerevisiae* and *E. coli*, respectively. Transcription of PPS1 mRNA is driven by the pGAL1 promoter. Transcription from this promoter is low during growth on dextrose as carbon source and is induced upon a shift to galactose as carbon source (21). Since the PPS1 sequence used to construct plasmid pBE33 includes only the PPS1 coding sequence, translation initiation is driven by the ADE1 translational initiation sequence (22) present in the pYES2AT vector. Vectors directing the expression of mutant proteins C478S and C525S were similarly constructed. 

**Plasmid Construction**—A 5.9-kb fragment of plasmid pBE24 containing the pps1A allele along with flanking sequence (Fig. 1B) was gel-isolated after EcoRI SalI digest of this plasmid. This fragment was transformed into strain GYC86, with transformants selected for uracil prototrophy. Ura- transformants were sporulated, and the resulting haploid strains were scored for mating type and markers known to be present in the parent strain.
Preparation of Yeast Chromosomal DNA and Southern Hybridization—Yeast DNA was prepared by the method of Guan et al. (11). 6 µg of DNA was digested with HindIII, separated on a 0.9% agarose gel, and transferred to Nitran membrane (Schleicher and Schuell). Southern hybridization was carried out with random primed NH2-terminal probe (Fig. 1C). Hybridization conditions were the same as those used for the library screen, except that the filter was washed in 0.25 × SSC, 0.1% SDS at 65 °C prior to autoradiography.

Site-directed Mutagenesis and DNA Sequencing—Plasmid pBE22 was used as the substrate for primer-directed mutagenesis using the Bio-Rad phagemid mutagenesis system. The following primers were used: 5'-GAAGTTCTGGTGATCTTAGTCGAGGAGAT (C725S) and 5'-AAAGTCTCTGATCTATTGTCGGAGTCCTC (C725S). Both mutations were sequenced using the dyeideoxy method (Sequenase kit; U.S. Biochemical Corp.). The sequence of the entire PPS1 open reading frame was determined using automated dye terminator sequencing (Applied Biosystems).

Bacterial Expression, Purification, and Activity Assays—Plasmid pBE25, which contains wild type PPS1 sequence, was transformed into E. coli strain BL21/DE3. GST-PPS1 fusion protein was purified using a modification of the method of Guan and Dixon (20). Overnight cultures (60 ml) were grown at 30 °C. These cultures were diluted 1:100 into 6 liters of fresh medium and grown at room temperature for a further 6 h, until an A600 of 1 was reached. Expression of the GST-PPS1 fusion protein was induced by the addition of isoprpyl β-D-thiogalactopyranoside to a final concentration of 200 µM. The culture was incubated aerobically for a further 15 h (overnight).

Cells were harvested by centrifugation and washed with 600 ml of phosphate-buffered saline (PBS; 150 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4, pH 7.3). After the wash step, cells were resuspended in 80 ml of PBS containing 0.1% Triton X-100. 25 µl of 1 M dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 0.2 mM L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone. Cells were lysed by three passages through a French pressure cell at 4 °C, 1200 p.s.i. The resulting lysate was centrifuged at 15,000 × g for 15 min to remove the insoluble fraction. 50 ml of supernatant was incubated with 30 ml of 50% (v/v) glutathione-agarose beads at 4 °C with gentle shaking. The beads were washed five times with 30 ml of PBS, and fixed in 70% ethanol overnight. After fixation, the cells were collected and resuspended in 0.5 ml of sodium citrate, pH 7.0, and sonicated briefly to disperse clumps. RNAse A was added to a final concentration of 1 µg/ml and the samples were incubated at 50 °C for 1 h. Cells were washed again with PBS and stained with propidium iodide (50 µg/ml) for 1 h at room temperature. Cell clumps were again dispersed by brief sonication immediately prior to flow cytometry analysis. Flow cytometry was carried out in a Coulter Epics Elite flow cytometer.

Mutlicopy Suppressor Screen—A strain carrying the PPS1 overexpression plasmid (pBE33) was transformed with a YEp13-based S. cerevisiae genomic library (28). Transformants were selected on SC medium lacking uracil and leucine (SC - Ura, - Leu) with dextrose as carbon source. A total of 65,000 colonies were screened for suppression of the PPS1 overexpression arrest by replica plating on SC - Ura, - Leu medium that contained galactose as the carbon source. Under these conditions, cells carrying the pBE33 do not grow. Sixty colonies that grew under these conditions (MSP1, multicopy suppressor of PPS1 overexpression arrest) were selected for further study. Plasmids from these colonies were amplified in E. coli and subjected to another multicopy suppressor screen. Twenty-six colonies contained plasmids that were MSP+ for two successive screens. Single library plasmids were isolated from these colonies, confirmed as MSP+, and sequenced to determine the identity of the insert.

RESULTS

Identification and Analysis of the PPS1 Gene and Protein Sequence—A search of S. cerevisiae sequence in the GenBank data base (29) with the PTPase active site signature, resulted in the identification of a putative PTPase open reading frame. This sequence, named PPS1, is present in GenBank accession X76053, a 32,420 base pair segment located on the right arm of S. cerevisiae chromosome II (31). Open reading frames for the DBP3, formamidase, 1 mg/ml bovine serum albumin, 400 mM sodium phosphate buffer, pH 7.1, 5% SDS, and 100 µg/ml E. coli tRNA at 42 °C. Double-stranded probes complementary to DBP3 and ACT1 messages were generated by random-primed labeling of restriction digests of plasmids pBE22 and pGEMΔact (27), respectively. Band intensities, representing the relative abundance of each transcript, were measured using a Molecular Dynamics PhosphorImager.

Flow Cytometry Analysis—Yeast strains were grown as described above. 1 ml samples were harvested by centrifugation, washed with 1 ml of PBS, and fixed in 70% ethanol overnight. After fixation, the cells were collected and resuspended in 0.5 ml of sodium citrate, pH 7.0, and sonicated briefly to disperse clumps. RNAse A was added to a final concentration of 1 µg/ml, and the samples were incubated at 50 °C for 1 h. Cells were washed again with PBS and stained with propidium iodide (50 µg/ml) for 1 h at room temperature. Cell clumps were again dispersed by brief sonication immediately prior to flow cytometry analysis. Flow cytometry was carried out in a Coulter Epics Elite flow cytometer.

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**S. cerevisiae PPS1 Gene**

*Fig. 2.* A, nucleic acid and predicted amino acid sequence of the PPS1 open reading frame. Numbering of base pairs (left) and amino acids (right) begins at the putative initiator codon. Approximate boundaries of the NH2-terminal extension, phosphatase-like domain, and phosphatase domain are shown, and the two active site-like regions of PPS1p amino acid sequence are boxed. B, six *S. cerevisiae* protein-tyrosine phosphatases. PTPase domains were defined using the Pileup program in the Wisconsin Genetics Computer Group software package. PTPase domains consist of approximately 250 amino acids and contain the active site signature sequence HCXXGXXX(S/T). In this schematic representation, phosphatase domains are denoted by outline boxes, with the location of the active site signature sequence marked by diagonal lines. The imperfect active-site-like sequence of PPS1p is marked with vertical lines. Unrelated sequence is represented by unboxed lines. The amino acid sequence for each active site signature is shown in the right column. C, Pileup alignment of five *S. cerevisiae* dual specificity PTPase domains. Aminoacyl residues that are identical or closely related in at least three of the five PTPase domains are boxed. Gene and protein names are given on the left, with PPS1 representing the PPS1p carboxy-terminal PTPase domain and PPS1* representing the phosphatase-like domain.

**A**

| 1  | ATG GCT TCG GAA GGG CCA TCA AGA GAG TGG CTG CAC GAT GAT GGG  |
|----|---------------------------------------------------------------|
|    | MV LE VPS ITP FG E LHD L                                     |
| 2  | ATG CCA CTA CAT CAT GAG CAA GAA TGG CTT GAA TAA AAA AAG AGT  |
|    | MR LE HDQ DASH WPE CE E K F                                     |
| 32 | CTC GGT CAT CAT ATT TCG GTT GCA CCA CCA GAT TTT GCT CAT        |
|    | PW AHD IFS PQ CQ D PPH                                     |
| 145| TCT GTC GCA ATA GTC AAA TGG TCA GAT GAT AAT TCG GGT ATT       |
|    | SL AIV K S Q S D AHS N S A L                                  |
| 64 | TGG GAT ATT TCG GCA GAT AAT GTC AGC TAC CAG TCG TGG AAA ATT   |
|    | LR N S L E V N D I F Q S W K Y                                |
| 289| CTC ACT TCG TTT CAT AGA GAG GGC GAT ACC GAT GAA ACT GAT        |
|    | STFS RHE G TD G TCT GTG N 96                                  |

**B**

- S. cerevisiae PPS1 Gene

**C**

- S. cerevisiae PPS1 Gene

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C-terminal PTPase domain contains a good match to the PTPase active site signature, including all aminoacyl residues invariant across known PTPases. This domain includes the sequence identified in our original GenBank™ search. The active site region of the upstream PTPase-like domain, on the other hand, is a poor match to the PTPase consensus. This active site region lacks the histidyl and arginyl residues shown to be invariant across PTPases (32) and is predicted to lack PTPase activity. Nonetheless, the upstream PTPase-like domain shows convincing sequence similarity to other PTPase domains across its entire length (Fig. 2C) and, when used to query sequence data bases, produces PTPases among the search results. The amino-terminal extension of PPS1p consists of about 300 amino acids and does not show significant sequence similarity to any other known DNA or protein sequence.

Isolation and Initial Characterization of the PPS1 Gene—
The PPS1 gene was isolated using colony filter hybridization to identify a plasmid containing PPS1 sequence from a S. cerevisiae chromosomal library. The clone contained a 12,500-bp insert that included the complete sequence of the PPS1 and DPB3 genes. A 3561-base pair fragment of this library clone containing the entire PPS1 coding sequence, along with 433 bp preceding the presumptive ATG initiator codon and 709 bp of 2424 nucleotides.

Fig. 2—continued
downstream of the terminator codon, was ligated into plasmid pUC119. This subclone, pBE22, was used as the substrate for subsequent manipulations of PPS1 sequence. Sequence of the PPS1 open reading frame was determined using automated dye terminator sequencing. The sequence determined in this study was identical to that reported in GenBank accession number U72800.

Subsequent manipulations of pUC119. This subclone, pBE22, was used as the substrate for downstream of the terminator codon, was ligated into plasmid pUC119. This subclone, pBE22, was used as the substrate for subsequent manipulations of PPS1 sequence. Sequence of the PPS1 open reading frame was determined using automated dye terminator sequencing. The sequence determined in this study was identical to that reported in GenBank accession number U72800.

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mone or increased osmolarity was also undertaken. Poly(A)^+ mRNA was isolated from liquid cultures before and at several time points after the addition of either 0.9 M NaCl or α-factor. The mRNA was separated on an agarose gel, blotted, and hybridized to a double-stranded PPS1 probe. Like the single-stranded probe, the double-stranded probe hybridized to a band of approximately 2.7 kb. No significant change in the abundance of the PPS1 transcript was noted between cells grown in rich and minimal medium or in response to the addition of 0.9 M NaCl or α-factor (data not shown). These experiments indicate that the signaling pathways controlling the mating response and the response to medium osmolarity are intact in strains lacking PPS1p function. The apparently normal responses of pps1Δ strains to pheromone and osmolarity suggest that PPS1p does not act to dephosphorylate the MAP kinase family members FUS3p or HOG1p, activated as part of the mating and osmolarity responses, respectively (4, 5), but it remains possible that PPS1p is a redundant regulator of one or both of these pathways or indeed of another MAP kinase signaling pathway.

Expression and Purification of the PPS1 Protein—Plasmid pBE25 contains glutathione S-transferase coding sequence fused to the PPS1 open reading frame, with transcription driven by the E. coli tac promoter. E. coli cells containing this plasmid were grown at room temperature, and protein expression was induced over a relatively long period (15 h) with low levels of isopropyl β-D-thiogalactopyranoside. We found that other conditions, such as growth at 37 °C, higher levels of isopropyl β-D-thiogalactopyranoside, and shorter induction times led to the production of insoluble or inactive protein. GST-PPS1 fusion protein production was purified by glutathione affinity batch methods, and the GST domain was cleaved with thrombin. This step yielded a mixture of polypeptides, including PPS1p (predicted molecular mass, 91.7 kDa), thrombin, the GST domain, and several other polypeptides, as determined by SDS-polyacrylamide gel electrophoresis (Fig. 3, lane L). This mixture was further purified using an anion exchange step with a 0–600 m M NaCl gradient. The PPS1 protein was eluted from the column at approximately 275 m M NaCl (Fig. 3, lanes 47 and 48). Fractions containing homogenous PPS1p were pooled and assayed for phosphatase activity using p-NPP as a substrate. p-NPP activity was present only in fractions 48–51, with peak activity in fraction 47, in good agreement with the relative abundance of the PPS1 protein band in these fractions. Purified PPS1 protein was assayed at various pH values, ranging from 5.5 to 9.5. The pH optimum for the PPS1-catalyzed hydrolysis of p-NPP was determined to be 7.5. At pH 7.5, the \( K_m \) of PPS1p for p-NPP was measured at 13.4 mM. When assayed at this pH, using saturating concentrations of p-NPP, the turnover number was calculated at 0.24 s\(^{-1}\). This activity was inhibited by the presence of 1 mM sodium vanadate in the assay buffer. PPS1 protein was also able to inactivate human MAP kinase (ERK1p; Ref. 24), although a 1:1 mixture of MAP kinase and PPS1 protein resulted in only a 50% reduction in MAP kinase activity against myelin basic protein. The presence of 1 mM sodium vanadate in the MAP kinase inactivation assay buffer resulted in no inactivation of MAP kinase by the PPS1 protein. Three truncated glutathione S-transferase fusions of PPS1p were also expressed and partially purified. These truncations contained 1) the carboxyl-terminal 776 amino acids (both phosphatase domains and most of the NH2-terminal extension), 2) the carboxyl-terminal 493 amino acids (both phosphatase domains), and 3) the carboxyl-terminal 249 amino acids (downstream phosphatase domain only). Glutathione-removed fusion proteins of each of these three constructs catalyzed the hydrolysis of p-NPP at similar rates, suggesting that the pNPP activity of PPS1 resides in the most C-terminal phosphatase domain.

Mutation of Catalytic Cysteiny1 Residues and Peptide Dephosphorylation Assays—Experiments with truncated PPS1 proteins indicated that phosphatase activity is contributed by the most C-terminal phosphatase domain. To confirm this result, the putative catalytic cysteine residue (12) in each domain was changed to a seryl residue. Mutant proteins were purified using the methods developed for purification of the wild type protein and were assayed for p-NPP hydrolysis and MAP kinase inactivation. The results of these experiments support the hypothesis that the downstream phosphatase domain is active while the upstream phosphatase-like domain is largely inactive. The C478S mutant protein, which contains an un disrupted downstream phosphatase domain, showed a pH optimum, \( K_m \) for p-NPP, and turnover number indistinguishable from that of the wild type protein. In contrast, the C725S mutant protein had a turnover number of approximately 0.001 s\(^{-1}\), less than 0.5% of that of the wild type protein. Furthermore, while the wild type and C478S mutant proteins were able to inactivate human MAP kinase as described above, the C725S mutant protein showed no activity in this assay.

Denu and co-workers (25) have shown that the human dual specificity phosphatase VHR is capable of dephosphorylating both phosphothreonyl and phosphotyrosyl residues in a dephosphorylated synthetic peptide containing human MAP kinase sequence. In these studies, VHR rapidly dephosphorylated the
Like VHR, PPS1p shows a preference for phosphotyrosyl over phosphothreonyl residues. Unlike VHR, where these rates differ by about 2000-fold, PPS1 acts to dephosphorylate phosphothreonyl residues at a rate about 50–100-fold lower than the rate of phosphotyrosyl dephosphorylation. The PPS1p C478S mutant protein showed a time course of dephosphorylation similar to that of the wild type enzyme, while the PPS1p C725S showed a rate of phosphothreonyl dephosphorylation less than 1% of that of the wild type enzyme.

PPS1 Transcript Abundance Fluctuates during the Cell Cycle—The PPS1 open reading frame lies adjacent to the open reading frame of the DBP3 gene, a subunit of DNA polymerase II (Ref. 36; Fig. 1A). DBP3 was isolated by Araki et al. (36) using an antibody screen of a S. cerevisiae expression library. dbp3Δ mutants are viable but show a mutator phenotype. Strains carrying the dbp3Δ allele show a 2–6-fold increase in the reversion rate of point mutations, suggesting that the DBP3p subunit may be involved in maintaining fidelity of DNA replication. These workers also showed that the DBP3 transcript abundance fluctuates during the cell cycle, in a pattern similar to that shown by other transcripts whose products are involved in DNA replication (36). The divergent arrangement and proximity of the DBP3 and PPS1 open reading frames are consistent with possible coordinated regulation of expression of these genes.

Poly(A)^+ RNA was purified from synchronized cultures of strain GYC121 (PPS1). Cultures were grown to an A_{600} of approximately 0.5. At this time, α-factor was added, and the culture was allowed to enter growth arrest. Cells were viewed microscopically to confirm that the culture consisted of at least 90% unbudded cells, indicating that α-factor arrest had occurred. Cells were pelleted and resuspended in prewarmed medium in the absence of α-factor, allowing synchronized growth to begin. The culture was sampled at 15-min intervals, and poly(A)^+ RNA was isolated from each sample. The doubling time of the synchronized culture was measured at 78 min. Northern transfers of these samples were probed with random primer-labeled probes specific for DBP3, PPS1, and actin. DBP3 and PPS1 transcript levels were normalized to the intensity of the actin transcript, which did not fluctuate during the cell cycle.

The results of this experiment are shown in Fig. 5. The relative abundance of the PPS1 transcript during the cell cycle is very similar to that of DBP3. Since DBP3 is known to be cell cycle-regulated, with transcript abundance peaking in early S phase (36), the PPS1 transcript also peaks in early S phase. This pattern of expression supports the hypothesis that DBP3 and PPS1 are coregulated and suggests a role for PPS1p in the regulation of some aspect of DNA synthesis, the major process involved in the regulation of passage into or out of S phase. Alternatively, the PPS1 protein might be involved in the regulation of passage into or out of S phase. Since genetic deletion of PPS1 did not lead to a detectable phenotype, we could not use these pps1Δ mutants to help elucidate the role of PPS1. In a complementary approach designed to help distinguish the role of PPS1, we overexpressed the PPS1 protein in S. cerevisiae.

Overexpression of PPS1p Is Deleterious and Leads to Abnormal DNA Synthesis—Plasmid pBE33, a plasmid designed to overexpress the PPS1 protein in S. cerevisiae, contains a 2μ origin of replication, a URA3 selectable marker, the PPS1 gene under the control of the pGAL1 promoter, and translation initiator sequences from the ADE1 gene. Strains GYC86, GYC121, BE240 (GYC121/pBE33), and BE239 (GYC121/pYES2AT (no insert)) were grown in liquid culture with dextrose as the carbon source overnight and diluted 1:500 into fresh medium in the presence of galactose to activate transcrip-

![Fig. 4. Dephosphorylation of a synthetic diphosphorylated peptide by PPS1p. Assays contained 1.25 mM synthetic diphosphorylated peptide (-(pT)p(pY)-) and 3 μg wild type (A), C478S (B), or C725S (C) PPS1 protein. After incubation for the indicated time, the reaction was stopped, and reaction products were analyzed. In addition to the diphosphorylated peptide (black peaks), the di-
To Northern blot of poly(A) 

...features.

tion from the pGAL1 promoter. After 8 h of growth on galactose, strains GYC86, GYC121, and BE239 were growing normally, with doubling times of approximately 100–110 min, while strain BE240 was not detectably growing (doubling time >1000 min). Microscopic examination of these cultures (Fig. 6) revealed that strains GYC121 and BE239 were 70–75% budded, the normal state for a growing culture of S. cerevisiae. Strain BE240, the PPS1p-overexpressing strain, on the other hand showed only 11% budded, consistent with a synchronized arrest of this culture.

To confirm that this arrest was a specific consequence of the overexpression of PPS1p, we examined the growth rate and percentage of budded cells in several control cultures. Strain BE240 showed no abnormalities in growth rate or the percentage of budded cells when grown on dextrose, where transcription from the pGAL1 promoter is not activated. Furthermore, expression of catalytically inactive PPS1p (C725S) or of a related dual specificity protein phosphatase (YVH1p) from the same expression vector did not lead to noticeable changes in the growth rate or morphology of the overexpressing strain, supporting the specific nature of the effects of the overexpression of PPS1p. Finally, similar phenotypes were seen with three independent isolates of strain BE240, indicating that the synchronous arrest phenotype is not a result of a mutation occurring in the vector construction or transformation procedures.

The level of PPS1p overexpression in strain BE240 was examined using antibody methods. Total soluble protein from various strains was separated on denaturing acrylamide gels and transferred, and the resulting blots were probed with PPS1 antisera. PPS1p was detectable at wild type levels of expression and was absent in pps1Δ mutant strains. Three independent experiments indicated that PPS1p levels in strain BE240 were increased 15–30-fold after 2 h of growth in the presence of galactose and 8–10-fold after 8 h. This moderate level of PPS1p overexpression is compatible with the observed arrest occurring as a consequence of specific interaction between overexpressed PPS1p and its physiological substrate.

To further characterize the phenotype of PPS1p-overexpressing strains, the above strains, GYC86, GYC121, BE240 (GYC121/pBE33), and BE239 (GYC121/pYES2AT (no insert)), were grown overnight in medium containing dextrose and diluted 1:500 into medium containing galactose, as described. After the growth arrest, phenotypes were confirmed, and the cells were fixed and stained with propidium iodide. The cells were mononucleate when viewed under a fluorescence microscope. The cells were then subjected to flow cytometry analysis (Fig. 7). Haploid strain GYC121 showed the expected fluorescence peaks at 1n and 2n DNA content; diploid strain GYC86 showed peaks at 2n and 4n. Strain BE239, like the parent strain GYC121, showed peaks at 1n and 2n (data not shown). Strain BE240, the induced PPS1p overexpressor, showed a markedly different fluorescence intensity pattern. This strain showed a broad peak of cells with DNA content approaching 2n. Some cells contained amounts of DNA greater than 2n, indicating that these cells may have undergone more than a single round of DNA replication. These results, along with the expression pattern of the PPS1 transcript, are consistent with a role for PPS1 in maintaining S phase. In normal cells, the transcript is most abundant during S phase and decreases 2–3-fold as the synchronized culture enters G2 phase. When PPS1p is overexpressed, the cells exhibit an inability to exit S phase, as indicated by the high concentration of DNA in each cell.

**Multicopy Suppression of the PPS1p Overexpression Arrest**—The growth arrest of PPS1p overexpressors allowed us to select for genes that, when present on multicopy vectors, overcome the defect that leads to this arrest. We would expect that kinases and their regulators would be among the genes identified with this screen, since increased expression of this class of molecules might counteract the effects of increased levels of phosphatase expression. Library plasmids were transformed into a strain carrying the PPS1p overexpression vector and screened for a multicopy suppression of PPS1p overexpression (MSP⁺) phenotype. MSP⁺ plasmids were then sequenced to identify the inserted DNA. Of the 26 plasmids that were found to confer an MSP⁺ phenotype in two successive rounds of screening, 11 contained inserts that included the open reading frame for the RAS2 gene. In *S. cerevisiae*, RAS2p is a well
known regulator of adenylate cyclase (37), with increased RAS2 activity leading to increased intracellular cAMP levels, activation of the cAMP-dependent protein kinases TPK1p, TPK2p, and TPK3p, and ultimately to the breakdown of storage carbohydrate (38). We tested the ability of other molecules involved in this pathway to suppress the PPS1p overexpression defect. Neither the addition of 5 mM cAMP to the growth medium nor the presence of RAS1, TPK2, or TPK3 on a high copy vector restored the ability of PPS1p-overexpressing strains to grow on galactose. Taken together, these results suggest a role for PPS1p in regulation of a pathway involving RAS2p but not RAS1p, adenylate cyclase, or the cAMP-dependent protein kinases. To confirm that addition of cAMP to the growth medium had the expected effect on the accumulation of storage carbohydrates in these strains, we carried out iodine staining of cells grown in the presence or absence of cAMP (Table II). The results of these experiments show that exogenous cAMP has the expected effect on the accumulation of storage carbohydrates. No strain showed dark iodine staining when grown in the presence of 5 mM exogenous cAMP. In contrast, cells carrying empty vector or RAS2 in multicopy showed dark iodine staining, while cells carrying TPK1 or TPK2 in multicopy did not stain. These observations confirm that the presence of the TPK genes has the expected effect on the accumulation of storage carbohydrates and show that the presence of RAS2 in multicopy does not lead to decreased levels of carbohydrate in this strain.

DISCUSSION

Coregulation of PPS1 and DPB3—The structure of the PPS1-DPB3 region of chromosome II suggested possible coregulation of these genes, and this was confirmed by measurement of transcript abundance. Coregulation of other divergently transcribed genes has been shown to occur in S. cerevisiae. The best studied example of this type of regulation is the promoter region that directs the expression of the GAL1 and GAL10 genes (39). This promoter region contains both positive and negative regulatory elements that allow coordinated activation or repression of GAL1 and GAL10. Since both of these genes specify proteins involved in the catabolism of galactose, it is reasonable that their regulation would be coordinated. Similarly, the sporulation-specific genes SPS18 and SPS19 are divergently transcribed and coregulated. These genes were isolated independently and subsequently shown to share promoter elements (40). Although these two genes are coregulated, the SPS18 gene is transcribed at about four times the rate of transcription of the SPS19 gene. We see a similar pattern of expression of DPB3 and PPS1. Although the abundance of these transcripts fluctuates in concert during the cell cycle, the DPB3 transcript is more abundant than the PPS1 transcript at all stages of the cell cycle.

Nature of Defects Caused by PPS1 Overexpression—Having carried out an analysis of the phenotype of pps1Δ mutants without discovering any set of conditions where the pps1Δ allele is deleterious, we investigated the effects of overexpression of PPS1p. As noted, PPS1p overexpression leads to two major defects; cells overexpressing PPS1p arrest in a synchronous fashion that is morphologically similar to a G1 arrest, and these cells contain a G2 amount of DNA, as determined by flow cytometry analysis. These consequences of PPS1p overexpression appear to be contradictory. Cells that are arrested in G2 would be expected to be unbudded and to have a G1 amount of
DNA. Conversely, cells that contain a G2 amount of DNA would not be expected to be in an unbounded state. The trivial explanation of these two defects postulates that they are unrelated. It is possible that either the apparent G1 arrest or the G2 amount of DNA per cell represents the consequence of an event normally mediated by PPS1p, while the other is an overexpression artifact. It should be noted, however, that overexpression of the related YVH1p phosphatase in otherwise isogenic strains did not lead to any detectable phenotype and that a relatively modest 15-fold overexpression of PPS1p is sufficient to induce cells to arrest. Another possibility is that the PPS1p phosphatase has multiple functions during the cell cycle and that overexpression causes defects at each point of action. These phenotypes of PPS1p overexpressors are similar to those shown by S. cerevisiae cells expressing Cdl1, a human protein phosphatase isolated on the basis of its interaction with human cyclin-dependent kinases (41). Gyuris and co-workers (41) report that expression of this PTPase from the pGAL1 promoter results in a morphology similar to G1 arrest as well as nuclear abnormalities. In HeLa cells, Cdl1 transcript abundance was shown to peak in early S phase. Although Cdl1p and PPS1p do not share outstanding sequence similarity, we suggest that these enzymes may have similar roles in regulating progression through the cell cycle in yeast (PPS1p) and humans (Cdl1p).

Taken together, the pattern of PPS1 expression (peak transcript abundance correlated with other transcripts involved in DNA synthesis) and the fact that some cells in the PPS1p-overexpressing culture appeared to have undergone more than one round of DNA replication suggest a role for the PPS1p phosphatase in maintenance of the DNA synthesis phase of the cell cycle. In this model, PPS1p activity maintains the dephosphorylated state of some substrate that, when phosphorylated, promotes exit from the synthesis phase of the cell cycle. Under conditions where PPS1p is overexpressed, this substrate remains in the dephosphorylated state, and exit from S phase is delayed. This model is supported by the observation that the deleterious effects of PPS1p overexpression require a catalytically active phosphatase.

**Suppression of PPS1p Overexpression Defects by RAS2**—The results of a multiplicity suppressor screen indicate that RAS2, but not RAS1, CAMP, TPK2, or TPK3, is able to suppress the PPS1p overexpression defect. Our results point toward a role for PPS1p in a pathway involving RAS2p but not adenylate cyclase. This conclusion is strengthened by the observation that the RAS2 multicopy suppression plasmid, while sufficient to overcome the PPS1p overexpression arrest, does not lead to decreased levels of storage carbohydrate, indicating that the adenylate cyclase-TPK pathway is not activated under these conditions. Morishita and co-workers (33) have reported genetic interactions between RAS2 and several genes, including the protein kinase genes DBF2, CDC5, and CDC15, that function during the mitosis phase of the cell cycle. We predict that the PPS1 protein phosphatase acts to down-regulate these kinases, their regulators, or their substrates during the DNA synthesis phase of the cell cycle. A summary of these predictions is shown in Fig. 8. In this scheme, PPS1p acts during S phase to dephosphorylate a substrate protein, which, when phosphorylated by cell cycle kinases, allows M phase to proceed. Overexpression of PPS1 leads to hypophosphorylation of the substrate protein and to an inability to exit S phase. The presence of RAS2 in multicopy does not activate the CAMP-TPK pathway but allows cell cycle kinases to phosphorylate the substrate protein and promotes normal cell cycle progression.

Alternative models of PPS1p-RAS2p interaction are also possible. The point of action of PPS1p has not been elucidated in this work, and future experiments will be directed at the identification of PPS1p substrates. In addition to the RAS2 gene, the multicopy suppressor screen identified the protein phosphatase gene PPH22 as a common multicopy suppressor of the PPS1p overexpression arrest. We are currently investigating the possibility that PPH22p is a regulator of PPS1p activity.

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The *PPS1* Gene of *Saccharomyces cerevisiae* Codes for a Dual Specificity Protein Phosphatase with a Role in the DNA Synthesis Phase of the Cell Cycle

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