Genomic Analysis of PIS1 Gene Expression

Mary E. Gardocki, Margaret Bakewell, Deepa Kamath, Kelly Robinson, Kathy Borovicka, and John M. Lopes*

Department of Biological Sciences, Wayne State University, Detroit, Michigan

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The Saccharomyces cerevisiae PIS1 gene is essential and required for the final step in the de novo synthesis of phosphatidylinositol. Transcription of the PIS1 gene is uncoupled from the factors that regulate other yeast phospholipid biosynthetic genes. Most of the phospholipid biosynthetic genes are regulated in response to inositol and choline via a regulatory circuit that includes the Ino2p:Ino4p activator complex and the Opi1p repressor. PIS1 is regulated in response to carbon source and anaerobic growth conditions. Both of these regulatory responses are modest, which is not entirely surprising since PIS1 is essential. However, even modest regulation of PIS1 expression has been shown to affect phosphatidylinositol metabolism and to affect cell cycle progression. This prompted the present study, which employed a genomic screen, database mining, and more traditional promoter analysis to identify genes that affect PIS1 expression. A screen of the viable yeast deletion set identified 120 genes that affect expression of a PIS1-lacZ reporter. The gene set included several peroxisomal genes, silencing genes, and transcription factors. Factors suggested by database mining, such as Pho2 and Yli044c, were also found to affect PIS1-lacZ expression. A PIS1 promoter deletion study identified an upstream regulatory sequence element that was required for carbon source regulation located downstream of three previously defined upstream activation sequence elements. Collectively, these studies demonstrate how a collection of genomic and traditional strategies can be implemented to identify a set of genes that affect the regulation of an essential gene.

Yeast has been an excellent model for the study of phospholipid biosynthesis (Fig. 1) (11, 12, 28, 33). Phosphatidylinositol (PI) is an essential phospholipid in all eukaryotic cells (3, 11, 12, 28, 33, 38, 54). In yeast, PI is synthesized de novo by the product of the PIS1 gene, PI synthase (18, 23, 24, 36, 53–56), and represents 12 to 27% of the total phospholipid composition (11, 12, 28, 33). In addition to a structural role, PI is a precursor of phosphoinositides, sphingolipids, and inositol polyphosphates (11, 12, 28, 33). PI and these metabolites are required for a diverse set of processes that include glycolipid anchoring of proteins (69), signal transduction (21, 58), mRNA export (57, 64–66), and vesicle trafficking (17). In spite of the importance of PI and its metabolites, relatively little is known about factors that regulate PIS1 expression.

Our understanding of the role of PIS1 expression in regulating PI synthesis is conflicted. One study reported that overexpression of the human PIS1 gene in COS-7 cells yielded a significant increase in PI synthase activity (25-fold) but a modest increase in PI levels (8.2%) (50). However, another report indicated that overexpression of the rat PIS1 gene in NIH3T3 cells yielded elevated levels of PI, PI-4,5-P2, and PI-3,4,5-P3 (19). PIS1 overexpression also decreased the doubling time of transformed cells and accelerated G1 progression (19). Consistent with the effect on G1 progression, cyclin D1 and cyclin E levels were elevated (19). Furthermore, Rous sarcoma virus-infected NIH3T3 cells and activated erbB2-transformed NIH3T3 cells also overexpress PIS1 and have elevated PI levels (37). Finally, specific inhibition of PI synthase activity using inostatycin reduces PI levels and inhibits induction of S phase (18, 36).

PI synthase is a membrane-associated enzyme that catalyzes the condensation of CDP-diacylglycerol and inositol to PI (23, 54) (Fig. 1). Disruption of the PIS1 gene results in lethality (54). Because PIS1 is essential, it is not entirely surprising that yeast cells do not extensively regulate PIS1 expression or PI synthase levels (2, 24, 25). PIS1 gene expression is not coregulated with the other phospholipid biosynthetic genes in response to inositol and choline (2, 11, 12, 28, 33) but is instead regulated by carbon source and oxygen. PIS1 expression is repressed in response to glycerol and aerobic conditions (2, 25). Promoter deletion analysis identified three upstream activation sequence (UAS) elements (UAS1 to UAS3) required for PIS1 gene expression (25). However, the element required for glycerol repression was not identified. The region that includes the UAS3 element also contains an upstream regulatory sequence (URS) that binds Rox1p to exert anaerobic regulation (25). The significance of the anaerobic regulation is evidenced by altered membrane composition. PI levels are elevated in cells grown anaerobically, and phosphatidylcholine (PC) and CDP-diacylglycerol levels are also affected by oxygen (25).

PIS1 gene expression is insensitive to inositol and choline; however, inositol does affect PI synthase activity. High levels of inositol increase the rate of PI synthesis because the Km of PI synthase for inositol (0.21 mM) is ninefold greater than the intracellular concentration of inositol (24 μM) (41). When cells are grown in inositol, PI levels double at the expense of phosphatidylserine synthase (CHO1 gene product), the first enzyme in the PC branch of phospholipid biosynthesis (Fig. 1) (41).

* Corresponding author. Mailing address: Department of Biological Sciences, Wayne State University, 5047 Gullen Mall, Detroit, MI 48202. Phone: (313) 993-7816. Fax: (313) 577-6891. E-mail: jlopes @sun.science.wayne.edu.
PIS1 transcript levels are established by an unusual combination of low transcription initiation rates and very stable mRNA. The PIS1 gene is expressed from a weak promoter that is comparable in activity to that of the GAL4, INO2, and INO4 transcription factor genes (1). However, the weakness of the PIS1 promoter is compensated by a stable transcript (half-life, 58 min) (1). In fact, the PIS1 transcript is among the most stable yeast mRNAs (34). The combination of low transcription initiation rates and high transcript stability may ensure that PIS1 transcripts change slowly in response to environmental changes.

In this study, we utilized a genomic strategy to identify genes that affect regulation of PIS1 expression. Analysis of the viable yeast deletion set (VYDS) identified several genes that affect PIS1 expression and regulation. We also examined the role of transcription factors defined by a “ChIP-on-chip” (chromatin immunoprecipitation-on-microarray chip) approach (62) on PIS1 expression. Last, we identified a URS needed for PIS1 repression in response to glycerol.

**MATERIALS AND METHODS**

Strains, media, and growth conditions. The Saccharomyces cerevisiae strains used in this study were BRS1001 (MATa ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1), BY4742 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), and BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and the complete VYDS in the BY4742 and BY4744 backgrounds. Yeast cultures were grown at 30°C in complete synthetic medium (42) (containing 2% glucose) lacking uracil. Where appropriate, glucose concentration was varied or substituted with 3% glycerol, 3% acetate, 3% ethanol, 3% lactate, or 0.1% oleic acid with 0.2% Tween 80. Where indicated, 75 μM inositol and 1 mM choline were added (1°C medium) and/or NaCl was added (0.7 M final concentration).

**Genomic studies.** Plasmid pMA107 (2), containing 629 bp of the PIS1 promoter fused to the lacZ gene, was transformed into the BY4742-based VYDS (Res Gen Invitrogen Corp., Carlsbad, Calif.) by a standard lithium acetate yeast transformation procedure (14) in 96-well microtiter plates. Transformants were selected on complete synthetic medium lacking uracil (Ura−), replicated onto Ura−% glucose X-Gal plates or a dark-blue phenotype on 2% glucose X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) and Ura−% glycerol X-Gal plates and allowed to grow at 30°C for 48 h. VYDS transformants were screened for altered PIS1-lacZ expression, indicated by a dark-blue phenotype on 2% glucose X-Gal plates or a dark-blue phenotype on 3% glycerol X-Gal plates relative to the isogenic wild-type (BY4742) strain. To eliminate false positives, pMA107 transformants that exhibited a phenotype transformed with plasmids pH330 (543 bp of the INO1 gene and 132 codons of the INO1 gene fused in frame to the lacZ reporter gene in Yep537R) (22) and Yep537R-PIS1-lacZ (described below). Mutant alleles in the VYDS were confirmed by sequencing bar codes that marked each mutant.

**Plasmid construction.** The construction of some of the PIS1-cat reporters has been previously reported (25). Briefly, a nested set of PIS1 promoter deletions was created by PCR using appropriate oligonucleotides (Table 1). The 5′-terminal deletion mutants were created using the reverse primer PIS1−81F to PIS1−127 (Table 1). The individual PCR products were cloned into pGEM-T (Promega, Madison, Wis.). PIS1 promoter fragments were excised from pGEM-T by digestion with BamHI and BglII and inserted into pBM2015 to create a fusion with the cat reporter gene (29). To create PIS1 promoter internal deletions, the regions from position 32 to 205 were amplified using forward primers (Table 1) and the reverse primer set PIS1−572F to PIS1−1. These fragments were sequentially cloned into pGEM-T and pBM2015 as described above. The regions downstream of each internal deletion were amplified using the reverse primer PIS1−805 to PIS1−572 followed by PIS1−1 and the forward primer set PIS1−101F to PIS1−101F in addition to PIS1−101F and PIS1−127 (Table 1). These fragments were sequentially cloned into pGEM-T and pBM2015 containing the relevant regions above the deletion endpoints as described above. For each plasmid, the name indicates the deletion endpoints. The pBM2015 derivatives were sequenced by the Wayne State University Core Sequencing Facility by using the primer pBM2015-SEQ. Yeast strains containing the promoter-cat plasmids integrated at the GAL4 locus were created by transformation and characterized by Southern blot hybridization as previously described (4).

**TABLE 1. Oligonucleotides used in this study**

| Oligonucleotide (position) | Sequence |
|---------------------------|----------|
| PIS1−3 (−1)               | GGATCCCGGATCCTTGATATTTTCAACAC-3′ |
| PIS1−918                   | AGATCTGGAATTCCCTGACGCG-3′ |
| PIS1−621                   | AGATCTTGATTTTCTAAGCCATG-3′ |
| PIS1−521                   | AGATCTGGATTAACCCATTTGCTTC-3′ |
| PIS1−325                   | AGATCTCGTGTCTTGGACTGTC-3′ |
| PIS1−224                   | AGATCTGTTTTTTCATTACCTTA-3′ |
| PIS1−205                   | AGATCTTGATTTTAAGTCTGCGC-3′ |
| PIS1−184                   | AGATCTTCTGTTATTTGAGGAA-3′ |
| PIS1−149F                  | AGATCTGGATTTTTTTCATTACCTTA-3′ |
| PIS1−138                   | AGATCTGTTTTTTCATTACCTTA-3′ |
| PIS1−127                   | AGATCTGTTTTTTCATTACCTTA-3′ |
| PIS1−185                   | GGATCTTCACGCGGAGGAGC-3′ |
| PIS1−149B                  | GGATCTTCACGCGGAGGAGC-3′ |
| PIS1−126B                  | GGATCTACGCGGGAGGAGC-3′ |
| PIS1−101B                  | GGATCTACGCGGGAGGAGC-3′ |
| PIS1−76B                   | GGATCTACGCGGGAGGAGC-3′ |
| PIS1−51B                   | GGATCTACGCGGGAGGAGC-3′ |
| PIS1−101F                  | GGATCTACGCGGGAGGAGC-3′ |
| PIS1−51F                   | GGATCTACGCGGGAGGAGC-3′ |
| PIS1−26F                   | GGATCTACGCGGGAGGAGC-3′ |
| PIS1−URS (−70)             | GGATCTACGCGGGAGGAGC-3′ |
| PIS1−URS (−51)             | GGATCTACGCGGGAGGAGC-3′ |
| PIS1−TCM1 (−1)             | GGATCTACGCGGGAGGAGC-3′ |
| PIS1−TCM1 (−1)             | GGATCTACGCGGGAGGAGC-3′ |

**FIG. 1. Schematic depiction of the S. cerevisiae phospholipid biosynthetic pathway.** The CDP-choline pathway, also known as the “Kennedy” and “salvage pathway,” is noted by a broken arrow. Genes are designated in boldface and italic type. Abbreviations: DHA, dihydroyxacetone; DHAP, dihydroxyacetone phosphate; PA, phosphatidic acid; DGPP, diacylglycerol pyrophosphate; DAG, diacylglycerol.
TCM1 gene fused in frame to the lacZ reporter gene in YEp357R (52). This plasmid was constructed by amplifying 572 bp of the TCM1 promoter using S. cerevisiae genomic DNA (Res Gen Invitrogen Corp.) by using primers TCM1 (−572) and TCM1 (−1) (Table 1). The 572-bp PCR product was cloned into pGEM-T (Promega) and then excised by digestion with HindIII and EcoRI and inserted into YEp357R. Insert orientation and sequence were confirmed by DNA sequencing.

Plasmid pLG312 + PIS1 URS51 contains a 25-bp fragment of the PIS1 promoter (−76 to −51) inserted upstream of the lacZ reporter gene in pLG312 (32). This plasmid was constructed by amplifying 25 bp of the PIS1 gene promoter (−76 to −51) from plasmid pPIS1(−325) using primers PIS1 URS (−76) and PIS1 URS (−51) (Table 1). The 37-bp PCR product was cloned into pGEM-T (Promega) and then excised by digestion with XhoI and inserted into plasmid pLG312. Insert orientation and sequence were confirmed by DNA sequencing.

**Enzyme assays.** β-Galactosidase and chloramphenicol acetyltransferase (CAT) assays were performed as described previously (4, 48). However, some of the β-galactosidase experiments were performed using a microtiter plate format. Units of β-galactosidase activity were defined as follows: (A420/minute/milligram of total protein) × 1,000. Units of CAT activity were defined as counts per minute in the organic phase and expressed as a percentage of the total counts per minute (percent conversion) divided by the amount of protein assayed (in micrograms) and the time of incubation (in hours). Protein concentration in each extract was determined by using a Bio-Rad (Rockville Center, N.Y.) protein assay kit. A minimum of five independent measurements were made for each data point.

**Growth phase assays.** Expression of the PIS1 gene was monitored from the expression of all three reporters (109 strains on glucose and 8 strains on glycerol) were removed from the data set. The remaining mutants affected PIS1 specifically, PIS1 and INO1, or PIS1 and TCM1 (Table 2).

In strains grown on 2% glucose, 25 mutant strains displayed decreased PIS1-lacZ expression, 20 mutant strains affected PIS1-lacZ and INO1-lacZ expression, and 24 mutant strains yielded altered PIS1-lacZ and TCM1-lacZ expression (Table 2). In strains grown in 3% glycerol, PIS1-lacZ expression was specifically altered in 20 mutant strains, 21 mutant strains had altered PIS1-lacZ and INO1-lacZ expression, and 10 strains yielded altered PIS1-lacZ and TCM1-lacZ expression (Table 2).

The mutant set contains an overrepresentation of mutants that affect a few specific biological processes. The genomic screen identified six genes involved in peroxisome biogenesis (pex3, pex4, pex17, and pex22) and function (acb1 and gpd1). The GPD1 gene is also involved in an early step in phospholipid biosynthesis (Fig. 1). The screen also yielded four genes

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### TABLE 2. Genes that affect PIS1-lacZ expression

| Reduced expression in glucose | Increased expression in glycerol |
|-----------------------------|---------------------------------|
| PIS1 | PIS1 and INO1 | PIS1 and TCM1 | PIS1 | PIS1 and INO1 | PIS1 and TCM1 |
| APT1 | APG17 | ACE2 | AAD4 | ACB1 | NYY1 |
| CDP1 | COQ4 | CIK1 | DC3 | ADH2 | PAC1 |
| CPT1 | DYNI | CPS1 | ENT4 | CTEF9 | RIF1 |
| CRH1 | ENT3 | DFG10 | HST3 | CVT19 | YDR445C |
| CWH41 | HIR3 | EAP1 | HST4 | EGD1 | YDR521W |
| FAB1 | PDX3 | GSH1 | OGG1 | HSP104 | YGL250W |
| GCS1 | REG2 | LS3 | PEX3 | NTG2 | YLL032C |
| GPDP | RMD1 | MUD1 | PEX4 | PEX22 | YLL054C |
| MUM2 | RP99 | NIX3 | PEX17 | PEX1 | YLL057C |
| NRG2 | RPS25A | PE9X | PMT3 | THI12 | YPL144W |
| OSM1 | VT1 | PHO2 | PTC3 | TIR4 | YPL032C |
| PET130 | YBR053C | PTC1 | ROT2 | VPS33 | |
| PIM1 | YDL119C | RP8B | RPL8B | VTS1 | |
| POGL | YDL129W | SIN3 | SMY1 | YPI1 | |
| RH54 | YDL173W | SIT4 | STM1 | YPI1 | |
| SP075 | YGL165C | TTR1 | YDR124W | YDR417C | |
| SRT1 | YLI178C | YAL068C | YGR022C | YOL153C | |
| WH14 | YNR025C | YDL025C | YKR043C | YOR315W | |
| YBR250W | YOL107W | YDL057W | YOR029W | YPL162C | |
| YDR323W | YPR123C | YDL063C | YOR322C | YPL166W | |
| YGL050W | YKL147W | YLR254C | YPR151C | YPR151C | |
| YOR242W | YLR254C | YLR360W | YPR151C | YPR151C | |
| YL028W | YLR360W | YNL134C | YMR073C | YNL134C | |
| YPL077C | YMR073C | YNL134C | YMR073C | YNL134C | |

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**RESULTS**

A genome-wide screen for genes that affect PIS1-lacZ expression in glucose and glycerol. To obtain a comprehensive understanding of the regulation of PIS1 expression, we screened the VYDS (~4,800 viable mutant strains) for changes in PIS1-lacZ expression of strains grown on different carbon sources. Even though PIS1 is regulated only threefold in response to carbon source, this regulation was clearly discernible on X-Gal medium containing glucose (dark blue) and glycerol (light blue). The PIS1-lacZ reporter (pMA107) (2) was transformed into the BY4742-based VYDS (MATa) set, and transformants were screened for light-blue colonies on X-Gal glucose medium and dark-blue colonies on X-Gal glycerol medium. Three rounds of screening identified 178 mutants that yielded reduced expression in glucose and 59 mutants with increased expression in glycerol. To eliminate mutants that nonspecifically affected lacZ expression, we rescreened the mutants using INO1-lacZ (phospholipid biosynthetic gene) (22) and TCM1-lacZ (ribosomal protein gene) reporters. Strains exhibiting altered expression of all three reporters (109 strains on glucose and 8 strains on glycerol) were removed from the data set. The remaining mutants affected PIS1 specifically, PIS1 and INO1, or PIS1 and TCM1 (Table 2).

In strains grown on 2% glucose, 25 mutant strains displayed decreased PIS1-lacZ expression, 20 mutant strains affected PIS1-lacZ and INO1-lacZ expression, and 24 mutant strains yielded altered PIS1-lacZ and TCM1-lacZ expression (Table 2). In strains grown in 3% glycerol, PIS1-lacZ expression was specifically altered in 20 mutant strains, 21 mutant strains had altered PIS1-lacZ and INO1-lacZ expression, and 10 strains yielded altered PIS1-lacZ and TCM1-lacZ expression (Table 2).

The mutant set contains an overrepresentation of mutants that affect a few specific biological processes. The genomic screen identified six genes involved in peroxisome biogenesis (pex3, pex4, pex17, and pex22) and function (acb1 and gpd1). The GPD1 gene is also involved in an early step in phospholipid biosynthesis (Fig. 1). The screen also yielded four genes
required for chromatin silencing (rif1, hst3, hst4, and sin3). There is an overrepresentation of mutants in DNA repair (pic3, ogg1, ntg2, rpb4, and rpb9) which has recently been associated with PI metabolism (84). It is interesting that two subunits of RNA polymerase involved in transcription-coupled repair were also identified (rpb4 and rpb9). The genomic screen also yielded two mutants involved in carbon source regulation that may play a role in glycerol repression (reg2) and also yielded two mutants involved in carbon source regulation that may play a role in glycerol repression (reg2). Last, genes required for PI metabolism were also identified (FAB1 and ACO1 are required for synthesis of phospholipidic and sphingolipids, respectively). We assume that the genomic screen described here did not identify every gene affecting PIS1-lacZ expression (see below); however, it did provide a wealth of information that would not have been possible if more traditional genetic screens were used.

**Peroxisomal biogenesis affects glycerol regulation of PIS1-lacZ expression.** Peroxisome biogenesis is a conserved process among eukaryotes, involving at least 32 known peroxins (20, 60, 70, 72–74, 77, 78, 80, 81). Peroxisomes are membrane-bound organelles that function in metabolic pathways involved in the inactivation of toxic substances (H2O2-based respiration), the regulation of cellular oxygen levels, and the metabolism of lipids, nitrogen bases, and carbohydrates including fatty acid β-oxidation (60, 72, 74, 77, 78). Genomic analysis of the VYDS strain collection identified that PIS1-lacZ expression in cells grown on 3% glycerol X-Gal plates was significantly increased in pexΔ, pex4Δ, pex17Δ, and pex22Δ mutant strains. However, we assumed that because the screen of the VYDS relied on a relatively modest phenotype, it probably did not identify all genes that regulate PIS1-lacZ expression. Given this possibility, we also tested the other known pexΔ mutants present in the VYDS that were not identified by the genomic plate screen. We performed liquid assays to quantify the effect of all known pexΔ mutants grown in medium containing 2% glucose and 3% glycerol on PIS1-lacZ expression. In addition, we also quantified PIS1-lacZ expression in medium containing 0.1% oleic acid, since this carbon source causes peroxisomes to proliferate (70).

As expected in the wild-type strain, PIS1-lacZ expression was repressed in 3% glycerol but unaffected in 0.1% oleic acid (Fig. 2). The results show that most of the pexΔ mutants affected expression of the PIS1-lacZ reporter under at least one growth condition. This included three of the mutants that were identified in the initial plate screen. That is, PIS1-lacZ gene expression in cultures grown in 3% glycerol was elevated in pex4Δ, pex17Δ, and pex22Δ mutant strains. It is important that we also observed elevated expression in the pex12Δ and pex13Δ mutants grown in 3% glycerol (Fig. 2). These two mutants were present in the original plate screen but eliminated by subsequent screens, suggesting that our screening criteria may have been too stringent. However, we also observed that the pex17Δ mutant identified in the plate screen did not reveal a difference relative to the wild type when quantified by the liquid assay. This discrepancy may be explained by the fact that the plate assay reports the accumulation of β-galactosidase over several phases of growth, whereas the liquid assay quantifies β-galactosidase activity at a single stage of growth.

The effect of the pexΔ mutants on PIS1-lacZ expression fell into four classes. Class I contained mutants that generally displayed the same pattern of PIS1-lacZ expression in the three carbon sources as that of the wild-type strain, elevated expression in glucose and oleic acid and decreased expression...
in glycerol (pex2Δ, pex3Δ, pex10Δ, pex11Δ, pex17Δ, and pex29Δ) (Fig. 2). However, the pex3Δ, pex10Δ, and pex11Δ mutants clearly yielded altered expression levels relative to that of the wild-type control. Class II included several mutants that yielded levels of expression in oleic acid that were below wild-type expression levels in glycerol and decreased levels of expression in oleic acid (pex4Δ, pex6Δ, pex12Δ, pex13Δ, pex14Δ, pex18Δ, pex22Δ, pex25Δ, pex27Δ, pex28Δ, and pex30Δ) (Fig. 2). Class III included mutants that yielded levels of expression in glycerol that were above wild-type expression levels in glycerol and decreased levels of expression in oleic acid (pex11Δ, pex12Δ, pex13Δ, pex14Δ, pex18Δ, pex22Δ, pex25Δ, pex27Δ, pex28Δ, and pex30Δ) (Fig. 2). Class IV included mutants that generally yielded low levels of expression in all three carbon sources (pex5Δ and pex7Δ) (Fig. 2).

The results clearly show that screening of the VYDS can yield valuable information regarding regulation of gene expression, even for a modestly regulated gene. These pex mutants could not possibly have been identified by more traditional means. Most importantly, these results provide the first evidence of coordination between the synthesis of peroxisomes and phospholipids.

Chromatin silencing genes affect PIS1-lacZ expression. Genomic analysis of the VYDS also identified rif1Δ, hst3Δ, and hst4Δ mutant strains with elevated PIS1-lacZ expression on 3% glycerol X-Gal plates. All three mutant strains are involved in chromatin silencing at telomeres. The HST3 and HST4 genes are homologs of Sir2 and are also involved in regulating short-chain fatty acid metabolism (8, 71). Hst3p is also required for silencing at the origin of replication from the endogenous 2μm plasmid (31). To determine if the PIS1-lacZ plate phenotype was due to genuine effects on transcription and not an effect on plasmid copy number, we assayed a PIS1-cat reporter stably integrated in single copy at the GAL4 locus. We also determined if mating type affected expression by comparing MATα reporters stably integrated in single copy at the GAL4 locus. We also determined if mating type affected expression by comparing CAT expression in MATα (BY4742 background) and MATα (BY4742 background) transformants. PIS1-cat expression in all three mutant strains was elevated in both glucose and glycerol regardless of mating type, although the hst3Δ and hst4Δ mutants had the most obvious effect (Fig. 3). These results also show that the plate phenotype was not due to an indirect effect of plasmid copy number. This is the first evidence of chromatin silencing affecting PIS1 expression.

Mutants in glycerol utilization affect regulation of PIS1-lacZ expression. Genomic analysis of the VYDS also identified the gpd1Δ mutant strain with decreased PIS1-lacZ expression on 2% glucose X-Gal plates. GPD1 encodes an NADH-dependent cytosolic glycerol-3-phosphate dehydrogenase, a key enzyme in the initial stages of glycerol metabolism (Fig. 1) (15, 61). This was an interesting result given that PIS1 gene expression is repressed by glycerol. This led us to determine if additional enzymes involved in the early stages of glycerol utilization also affect PIS1-lacZ expression. Thus, we quantified β-galactosidase activity in a gpd1Δ mutant strain as well as gpd2Δ, gut1Δ, and gut2Δ mutant strains grown in 2% glucose. GPD2 encodes an isofrom of glycerol-3-phosphate dehydrogenase (Fig. 1) (59), while GUT1 encodes glycerol kinase, and GUT2 encodes mitochondrial glycerol-3-phosphate dehydrogenase (Fig. 1) (26, 27).

PIS1-lacZ expression increased substantially in the gut2Δ strain (176%) and to a much lesser extent in the gut1Δ mutant strain (37%) when cells were grown in 2% glucose (Fig. 4). As expected from the VYDS screen, the gpd1Δ strain yielded a 20% decrease in β-galactosidase activity (Fig. 4). However, the gpd2Δ mutant strain was indistinguishable from the wild-type control (Fig. 4).

PIS1 promoter binding proteins identified by ChIPs are required for PIS1 expression. In an effort to identify additional factors that regulate PIS1 expression, we mined a ChIP-on-chip database containing information on yeast DNA-binding proteins (47). This analysis identified Pho2, Yfl044c, and Ste12 as weak candidate PIS1 promoter binding proteins (47). Pho2 is involved in the response to phosphate starvation (5, 39, 40, 43, 51, 67, 68), while Ste12 is involved in pheromone and pseudohyphal regulation (16, 35, 62, 83). YFL044c encodes a hypothetical open reading frame (ORF) with unknown function. Interestingly, the pho2 mutant was identified in our plate screen as yielding reduced expression on 2% glucose X-Gal medium (Table 2). Previously, three UAS elements (UAS1
PIS1-lacZ expression was assayed by using plasmid pMA107 containing 629 bp of PIS1 gene promoter (−629 to −1). Plasmid pMA107 was transformed into a wild-type (WT) strain (BY4742; hap1Δ, ura3Δ, his3Δ, leu2Δ, met15Δ, and his3Δ). Expression generally decreased in the three mutant strains, but the effect was most obvious in the yep3Δ mutant strain (Fig. 6A, compare pPIS-149 and pPIS-127). Prevention of UAS1 decreased PIS1-lacZ expression by half (Fig. 6A, compare pPIS-224 and pPIS-205), while deletion of UAS2 exacerbated the decrease in PIS1-lacZ expression by another 90% (Fig. 6A, compare pPIS-205 and pPIS-149). PIS1-lacZ expression was below detectable levels when UAS3 was also deleted (Fig. 6A, compare pPIS-225 and pPIS-127). However, the data showed that none of these UAS elements were required for the response to glycerol. Curiously, deleting any one of the three UAS elements increased the degree of repression caused by growth in glycerol (from ∼3-fold to ∼8-fold [Fig. 6A]). One explanation for this result is that when the UAS elements are deleted, the glycerol repression factor is more effective in repressing transcription because promoter activity is lower.

Because the three UAS elements are required for expression, it was necessary to generate internal deletions in order to analyze promoter sequences downstream of the three UAS elements. Analysis of the PIS1 promoter downstream of the three UAS elements identified a repressor site (URSGLY), located promoter proximal, required for glycerol repression. The data identified a 25-bp region (−76 to −51) that, when deleted, abolishes PIS1 glycerol repression (Fig. 6A). This organization of regulatory elements is reminiscent of GAL4 gene expression which is repressed fourfold by growth in glucose and requires a URS element located downstream of a UAS element (29, 30). A heterologous system was also employed to further examine PIS1 URSGLY function. The 25-bp region (−76 to −51) defined by the deletion analysis was inserted into plasmid pLΔ312, previously utilized to test URS function (32, 49). Plasmid pLΔ312 contains the CYC1 promoter fused to the lacZ reporter gene. The 25-bp pIS1 URSGLY caused CYC1-lacZ expression to decrease dramatically (Fig. 6B). This result indicates that the PIS1 URSGLY sequence functions as a URS element. The URSGLY effect on CYC1-lacZ expression in cells grown in glycerol could not be assessed because the CYC1 promoter is regulated by a carbon source.

Glycerol repression of PIS1 expression is reversed by glucose. Glycerol-mediated repression of PIS1 transcription is unusual since it does not appear to involve any of the previously identified regulators of carbon source regulation (1, 2). To determine if glycerol-mediated repression is reversible, PIS1-cat activity from a wild-type strain (BRS1001) containing pPIS-325 was assayed from culture grown in medium containing 2% glucose or 3% glycerol with various concentrations of YFL044c ORF (along with 5′- and 3′-flanking sequences) into the yfl044c mutant strain and assayed it for PIS1-lacZ expression. The presence of YFL044c on the pRS200 plasmid restored PIS1-lacZ expression to a level ∼3-fold higher than that of the wild-type strain (data not shown). This result suggests that Yfl044c may be limiting for PIS1 expression in a wild-type strain under the conditions tested here.

**PIS1 promoter deletion analysis identifies a regulatory element required for glycerol repression.** A nested set of deletions of the PIS1 promoter fused to the cat reporter gene in plasmid pBM2015 was transformed into a wild-type strain (BRS1001) targeting integration at the GAL4 locus in single copy and in a single orientation. We have previously reported the analysis of a deletion subset grown in glucose that identified three UAS elements at positions −224 to −205 (UAS1), −184 to −149 (UAS2), and −149 to −138 (UAS3) (25). Deletion of UAS1 decreased PIS1-lacZ expression by half (Fig. 6A, compare pPIS-149 and pPIS-205), while deletion of UAS2 exacerbated the decrease in PIS1-lacZ expression by another 90% (Fig. 6A, compare pPIS-205 and pPIS-149). PIS1-lacZ expression was below detectable levels when UAS3 was also deleted (Fig. 6A, compare pPIS-225 and pPIS-127). However, the data showed that none of these UAS elements were required for the response to glycerol. Curiously, deleting any one of the three UAS elements increased the degree of repression caused by growth in glycerol (from ∼3-fold to ∼8-fold [Fig. 6A]). One explanation for this result is that when the UAS elements are deleted, the glycerol repression factor is more effective in repressing transcription because promoter activity is lower.

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glucose (range, 0.1 to 2%). The data show that glucose concentrations as low as 0.1% were able to partially reverse glycerol repression and that concentrations from 1 to 2% completely reversed repression of \textit{PIS1-cat} expression (Fig. 7).

\textit{PIS1} expression is elevated in fermentable carbon sources (glucose and galactose) relative to a nonfermentable carbon source (glycerol) (2). We quantified the effect of other nonfermentable carbon sources on \textit{PIS1-cat} expression by using the \textit{pPIS-325} construct. In addition to glycerol, \textit{PIS1-cat} expression is reduced \textit{~50%} when cells are grown with acetate, ethanol, or lactate as a carbon source (Fig. 8).

\textit{PIS1} expression is not affected by growth phase or hyperosmotic stress. It has been reported that several phospholipid biosynthetic genes are regulated in response to growth phase (46). For example, \textit{CHO1-lacZ} expression levels in \( \Gamma^{-C^-} \) medium are low during lag phase and increase throughout log phase, peaking at the beginning of stationary phase (46, 63). Once in stationary phase, expression levels decrease precipitously until they reach the initial expression levels seen in lag phase. This expression pattern is similar in \( \Gamma^{-C^-} \) medium; however, because expression is repressed in this medium, the overall expression levels are lower than those observed in the \( \Gamma^{-C^-} \) medium (46, 63). We also discovered that growing cells in hyperosmotic medium (0.7 M NaCl) eliminates the growth phase regulation without affecting the inositol-choline-mediated regulation (63). These observations prompted an examination of the effect of growth phase and hyperosmotic conditions on \textit{PIS1} expression. This examination was done by quantifying CAT activity from \textit{pPIS1-918} transformants of \textit{BRS1001}. These experiments revealed that neither growth phase nor hyperosmotic medium affected expression of the \textit{PIS1-cat} gene (Fig. 9).

**DISCUSSION**

Until recently, \textit{PIS1} gene expression was not believed to be regulated (2, 25, 45, 75, 76). However, we have previously shown that \textit{PIS1} expression is regulated by carbon source (2) and in response to oxygen availability (25, 45, 75, 76). Multiple approaches were applied to identify genes that affect \textit{PIS1} expression. The genomic screen of the \textit{VYDS} identified 69 mutants with reduced \textit{PIS1} expression in glucose and 51 mutants with increased \textit{PIS1} expression in glycerol. \textit{PIS1} promoter deletion analysis coupled with mining of databases of...
DNA-binding proteins identified three UAS elements and one URS element and putative cognate transcription factors. For example, the promoter deletion analysis identified one UAS element with potential Ste12 and Pho2 binding sites (UAS1 [−224 to −205]), an additional UAS element with a potential Pho2 binding site and two known Mcm1 binding sites (UAS2 [−184 to −149]), and a potential Gcr1 binding site within another UAS element (UAS3 [−149 to −138]), as well as the Rox1 binding site essential for the anaerobic regulation of \( \text{PIS1} \) (25). Comparison of promoters from related \( \text{Saccharomyces} \) yeast species revealed strong sequence similarity among the three UAS regions, including the potential Ste12 binding sites, and known Mcm1 and Rox1 binding sites between \( \text{S. cerevisiae} \) and \( \text{S. castellii} \), \( \text{S. bayanus} \), \( \text{S. kluveri} \), \( \text{S. kudriavzevii} \), and \( \text{S. mikatae} \) (25). Mining of the yeast ChIP-on-chip database identified \( \text{Yfl044c} \) and Mcm1 as candidate \( \text{PIS1} \) promoter binding proteins (47).

This study demonstrated that genomic approaches provide an excellent means to identify regulatory mechanisms that control modestly regulated and essential genes such as \( \text{PIS1} \). However, while single strategies provide an excellent starting point, it is the combination of multiple genomic approaches that is essential. For example, Rox1 was not identified as a regulator of the \( \text{PIS1} \) promoter by the ChIP-on-chip study (\( P \) value of 0.19) (47) or by the VYDS screen; however, it was suggested by searches of databases of DNA-binding proteins coupled with promoter deletion (25) and microarray studies (45, 75, 76). Our previous studies have clearly shown that Rox1 binds and bends the \( \text{PIS1} \) promoter to exert anaerobic regulation on the \( \text{PIS1} \) gene (25). Conversely, the VYDS screen we employed here did not identify \( \text{Yfl044c} \), and examination of the \( \text{PIS1} \) promoter sequence does not reveal an obvious binding site for \( \text{Yfl044c} \) (TTCTTKTYTTTT) (47). However, the ChIP-on-chip study placed \( \text{Yfl044c} \) on the cusp of the acceptable binding threshold (\( P \) value of 0.006) (47), and we have shown here that \( \text{Yfl044c} \) does regulate \( \text{PIS1-lacZ} \) expression. The most obvious \( \text{PIS1} \) promoter binder defined by the ChIP-on-chip study was Mcm1 (\( P \) value of 0.000003), and this protein has
previously been shown to bind the **PIS1** promoter (44). However, the VYDS screen could not have identified **MCM1** since it is an essential gene. The use of multiple genomic approaches should also be expected to generate overlapping information. This was the case with Pho2 (also known as Bas2 and Grl10), which was suggested to be a regulator of **PIS1** by the promoter deletion analysis since potential binding sites are found in UAS1 and UAS2 (25). The VYDS screen also identified Pho2 as a regulator of **PIS1-lacZ** expression. However, the ChIP-on-chip database did not identify Pho2 as a potential binder (P value of 0.5) (47). These results underscore the benefits of employing multiple genomic screens coupled with database mining and more traditional approaches.

This study further demonstrated that genomic approaches might identify biological functions that affect the expression of a gene, thereby illuminating interplay between biological processes. The VYDS screen identified an overrepresentation of mutants that affect three biological processes: peroxisome biogenesis (**pex3, pex4, pex17, and pex22**) and function (**ach1** and **gpd1**), chromatin silencing (**rif1, hst3, hst4, and sin3**), and DNA repair (**ptc3, ogg1, mtg2, rp4b, and rp89**). Alterations of **PIS1** expression in the 26 assayed **pex** mutants are extremely interesting given the cellular role peroxisomes play, specifically in cellular oxygen regulation, and the metabolism of lipids, nitrogen bases, carbohydrates, and fatty acid β-oxidation (60, 72, 74, 77, 78). This finding suggests that changes within the peroxisome, potentially due to alterations in fatty acid metabolism (60, 72, 74, 77–79), could be responsible for alterations in **PIS1** expression and fatty acid metabolism. Our understanding of the role of Hst3 and Hst4 in silencing is not as well developed as that of other silencing proteins. Moreover, our studies cannot distinguish between direct and indirect effects on **PIS1** expression. Additional studies will focus on whether these proteins bind the **PIS1** promoter directly. The DNA repair genes defined in the VYDS screen included **RPB4** and **RPB9**. These genes may affect **PIS1** expression either because they play a regulatory role in transcription of **PIS1** or because of their role in DNA repair. Regardless of the mechanism whereby these two genes affect **PIS1** expression, it is clear that other DNA repair genes also affected **PIS1** expression. The identification of a link between a damage checkpoint pathway and PI metabolism has been suggested by other genome-wide studies (84). This is not entirely unexpected given that **PIS1** and PI have a role in cell cycle progression (18, 19, 36, 37).

The promoter deletion study identified a **URS** element, downstream of the three UAS elements, that was required for glycerol repression. Examination of the sequence defined by the **URS_{GAL}** element did not reveal any obvious protein-binding site. This observation is consistent with our findings that many genes involved in carbon source regulation in yeast do not affect **PIS1** expression (**REG1, GLC7, MIG1**, etc. [M. E. Gardocki and J. M. Lopes, unpublished results]). We did, however, find that **REG2** and **Nrg2** affected **PIS1-lacZ** expression and that these genes have a role in carbon source regulation (6). Nrg2 binds DNA directly, and its levels are decreased in glycerol- or ethanol-grown cells. However, Nrg2 is a repressor and would therefore be expected to increase **PIS1** expression in glycerol (6). Moreover, the **PIS1** promoter does not contain a binding site for Nrg2. Thus, it seems possible that a novel transcription factor may be involved in glycerol-mediated **PIS1** expression. This factor may be present in the collection of genes defined by the VYDS screen.

Our knowledge of **PIS1** expression, its regulation, and the various biological processes with which it may be intimately intertwined can only now begin to be discerned as a direct result of the results presented here. Genomic studies coupled with database mining and with more classical strategies can obviously be successfully combined to study modestly regulated genes. Of course, the challenge is in interpreting the wealth of information that these genomic approaches provide. A critical part of this process will be to distinguish between direct and indirect effects. Another important issue that will need to be addressed is to determine which of the genes and processes defined here affect PI metabolism. PI metabolism has been shown to be affected by anaerobic conditions via the Rox1 protein (25) and by carbon source (D. Kamath and J. M. Lopes, unpublished results), suggesting that regulation of **PIS1** expression does affect PI metabolism. Furthermore, the long half-life of the **PIS1** transcript (57 min) (1) and an AUG codon located upstream of the PI synthase AUG codon (25) may also contribute to the regulation of **PIS1** gene expression.

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