Genomic Analysis of *PIS1* Gene Expression

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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 Yfl044c, 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-P_2, and PI-3,4,5-P_3 (19). *PIS1* overexpression also decreased the doubling time of transformed cells and accelerated G_1 progression (19). Consistent with the effect on G_1 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

inostamycin 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 K_m 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 PC synthesis as inositol is also a noncompetitive inhibitor of phosphatidylserine synthase (*CHO1* gene product), the first enzyme in the PC branch of phospholipid biosynthesis (Fig. 1) (41).

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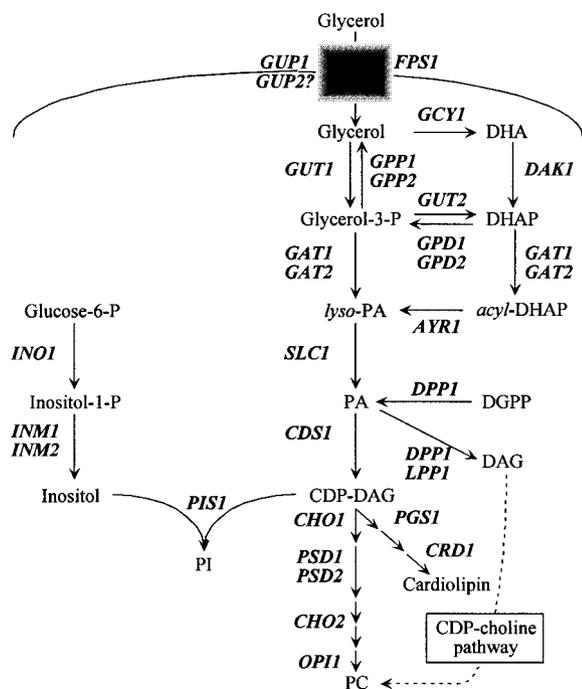
FIG. 1. Schematic depiction of the *S. cerevisiae* phospholipid biosynthetic pathway. The CDP-choline pathway, also known as the "Kennedy" and "salvage pathway," is noted by a broken arrow. Genes are designated in boldface and italic type. Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; PA, phosphatidic acid; DGPP, diacylglycerol pyrophosphate; DAG, diacylglcerol.

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 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), and BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and the complete VYDS in the BY4742 and BY4741 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 indi-

cated, 75 μM inositol and 1 mM choline were added (I^+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$^-$ 2% glucose X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and Ura$^-$ 3% 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 light-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 were transformed with plasmids pJH330 (543 bp of the *INO1* gene and 132 codons of the *INO1* gene fused in frame to the *lacZ* reporter gene in YEp357R) (22) and YEp357R-*TCM1* (*TCM1-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 PIS1-3′ (−1) along with the forward primer set PIS1 (−918) 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 −325 to various downstream points was amplified using forward primer PIS1 (−325) along with the reverse primer set PIS1 (−185) to PIS1 (−51B) (Table 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 PIS1-3′ (−1) along with the forward primer set PIS1 (−101F) to PIS1 (−26F) in addition to PIS1 (−149F) 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).

Plasmid YEp357R-*TCM1* contains 572 bp of the sequences upstream of the

TABLE 1. Oligonucleotides used in this study

Oligonucleotide (position)	Sequence
PIS1-3′ (−1)	5′-GGATCCCTTGTACTATCACAC-3′
PIS1 (−918)	5′-AGATCTGAATTCCCTGCACGC-3′
PIS1 (−621)	5′-AGATCTAAGCTTTTAGCCATG-3′
PIS1 (−521)	5′-AGATCTTAAATCCAAATCTTC-3′
PIS1 (−325)	5′-AGATCTTGTGCTTGAGGCTCA-3′
PIS1 (−224)	5′-AGATCTTTTTTTTCATTTTATA-3′
PIS1 (−205)	5′-AGATCTTTCACGTCGCTCCGC-3′
PIS1 (−184)	5′-AGATCTATCCCTATTACGGAA-3′
PIS1 (−149F)	5′-AGATCCGCCCCTCCTATTGTT-3′
PIS1 (−138)	5′-AGATCTGTTTTTTCCGTCTCG-3′
PIS1 (−127)	5′-AGATCTCTCGAGGATTTTTCA-3′
PIS1 (−185)	5′-GGATCCTTACGTGCGGAGCGA-3′
PIS1 (−149B)	5′-GGATCCTTTCCCTATTGAGAA-3′
PIS1 (−126B)	5′-GGATCCACGGAAAAAACAATA-3′
PIS1 (−101B)	5′-GGATCCATACTAAAAGTGAAA-3′
PIS1 (−76B)	5′-GGATCCCTCATGTTTTTTATG-3′
PIS1 (−51B)	5′-GGATCCTGACGGCAAATAAAC-3′
PIS1 (−101F)	5′-AGATCTATAAGTAAAACATAA-3′
PIS1 (−76F)	5′-AGATCTAGGTGGTATGGTTTA-3′
PIS1 (−51F)	5′-AGATCTCTGTGGAGCCTTCAA-3′
PIS1 (−26F)	5′-AGATCTTAAGAGGGAAAGTGT-3′
PIS1 URS (−76)	5′-CTCGAGAGGTGGTATGGTTTA-3′
PIS1 URS (−51)	5′-CTCGAGTGACGGCAAATAAAC-3′
TCM1 (−572)	5′-AAGCTTCTTACGTTATCATTC-3′
TCM1 (−1)	5′-GAATTCGATTGATTGTTGTAG-3′
pBM2015-SEQ	5′-TCTCGTGCGGAGATGACTGC-3′

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	NYV1
CPD1	COQ4	CIK1	DCP3	ADH2	PAC1
CPT1	DYN1	CPS1	ENT4	CTF19	RIF1
CRH1	ENT3	DFG10	HST3	CVT19	YDR445C
CWH41	HIR3	EAP1	HST4	EGD1	YDR521W
FAB1	PDX3	GSH1	OGG1	HSP104	YGL250W
GCS1	REG2	LSB3	PEX3	NTG2	YLL032C
GPD1	RMD1	MUD1	PEX4	PEX22	YLL054C
MUM2	RPB9	NHX1	PEX17	RIM8	YLL057C
NRG2	RPS25A	PEX7	PMT3	THI12	YPL144W
OSM1	VTA1	PHO2	PTC3	TIR4	
PET130	YBR053C	PTC1	ROT2	VPS33	
PIM1	YDL119C	RPB4	RPL8B	VTS1	
POG1	YDL129W	SIN3	SMY1	YTP1	
RDH54	YDL173W	SIT4	STM1	YCR076C	
SPO75	YGL165C	TTR1	YDR124W	YDR417C	
SRT1	YJL178C	YAL068C	YGR022C	YOL153C	
WH14	YNR025C	YDL025C	YKR043C	YOR315W	
YBR250W	YOL107W	YDL057W	YOR029W	YPL162C	
YDR332W	YPR123C	YDL063C	YOR322C	YPL166W	
YGL050W		YKL147W		YPR151C	
YGR242W		YLR254C			
YIL028W		YLR366W			
YMR073C		YNL134C			
YPL077C					

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 pLGΔ312 + *PIS1* URS$_{GLY}$ contains a 25-bp fragment of the *PIS1* promoter (−76 to −51) inserted upstream of the *lacZ* reporter gene in pLGΔ312 (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 pLGΔ312. 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 platform. Units of β-galactosidase activity were defined as follows: (A_{420}/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 BRS1001 transformants harboring the pPIS1-918 (*cat*) reporter construct. Cells were grown in I⁻C⁻ and I⁺C⁺ media in both the absence and presence of 0.7 M NaCl. Five-milliliter aliquots were removed at several intervals over the span of 100 h and monitored for density (using a Klett-Summerson colorimeter). CAT activity (described above) was determined from all samples simultaneously.

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 (*MAT*α 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
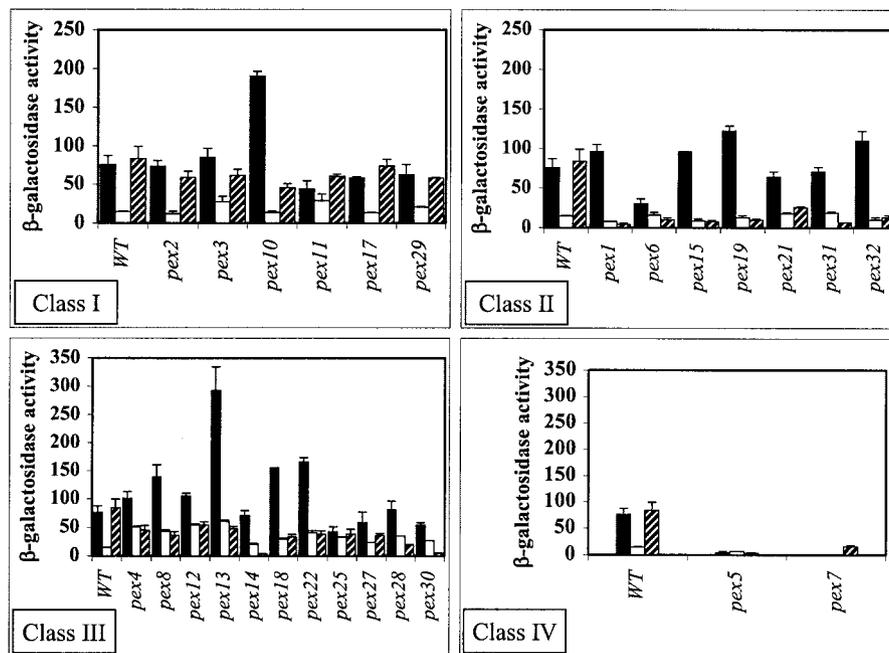
FIG. 2. Peroxisomal biogenesis mutants affect *PIS1-lacZ* gene expression. *PIS1-lacZ* gene expression was assayed by using plasmid pMA107 containing 629 bp of the *PIS1* gene promoter (−629 to −1). Plasmid pMA107 was transformed into a wild-type (WT) strain (BY4742; *MATα*) and isogenic strains containing *pexΔ* mutant alleles, and β-galactosidase activity was measured. Cultures were grown in Ura⁻ medium containing 2% glucose (solid bars), 3% glycerol (empty bars), or 0.1% oleic acid with 0.2% Tween 80 (hatched bars). The mutants were divided into four classes described in the text. Note that the *y*-axis range differs between the bar graphs.

required for chromatin silencing (*rif1*, *hst3*, *hst4*, and *sin3*). There is an overrepresentation of mutants in DNA repair (*ptc3*, *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 (*nrg2* and *reg2*). Last, genes required for PI metabolism were also identified (*FAB1* and *ACB1* are required for synthesis of phosphoinositides 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 (H_2O_2-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 *pex3Δ*, *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 *pex3Δ*, *pex4Δ*, 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 oleic acid (*pex1Δ*, *pex6Δ*, *pex15Δ*, *pex19Δ*, *pex21Δ*, *pex31Δ*, and *pex32Δ*) (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 (*pex4Δ*, *pex8Δ*, *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 CAT activity in *MAT***a** (BY4741 background) and *MAT*α (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 more 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 isoform 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
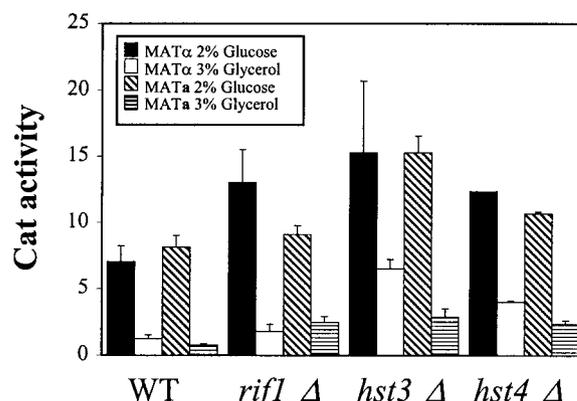

FIG. 3. Chromatin silencing gene mutants affect *PIS1-cat* expression. A *PIS1* promoter fragment (pPIS-325) was fused to the *cat* reporter gene and integrated in single copy at the *GAL4* locus in strains BY4742 (*MAT*α) and BY4741 (*MAT***a**) and isogenic *rif1Δ*, *hst3Δ*, and *hst4Δ* strains. *PIS1-cat* expression was assayed in transformants grown in medium containing either 2% glucose (*MAT*α, solid bars; *MAT***a**, hatched bars) or 3% glycerol (*MAT*α, empty bars; *MAT***a**, horizontal bars). WT, wild type.

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
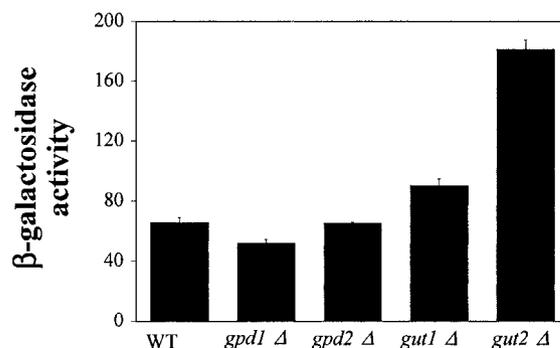

FIG. 4. Mutants in glycerol utilization affect regulation of *PIS1-lacZ* expression. *PIS1-lacZ* expression was assayed by using plasmid pMA107 containing 629 bp of *PIS1* gene promoter (−629 to −1). Plasmid pMA107 was transformed into a wild-type (WT) strain (BY4742; *MAT*α) and isogenic strains containing *gpd1Δ*, *gpd2Δ*, *gut1Δ*, and *gut2Δ* mutant alleles, and β-galactosidase activity was measured. Cultures were grown in Ura⁻ medium containing 2% glucose.
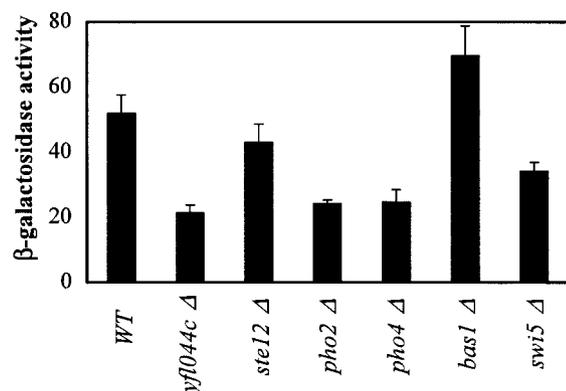
FIG. 5. Transcription factor mutants affect *PIS1-lacZ* gene expression. *PIS1-lacZ* expression was assayed by using plasmid pMA107 containing 629 bp of *PIS1* gene promoter (−629 to −1). Plasmid pMA107 was transformed into a wild-type (WT) strain (BY4742; *MAT*α) and isogenic strains containing *yfl044*Δ, *ste12*Δ, *pho2*Δ, *pho4*Δ, *bas1*Δ, and *swi5*Δ mutant alleles, and β-galactosidase activity was measured. Cultures were grown in Ura⁻ medium containing 2% glucose.

[−224 to −205], UAS2 [−184 to −149], and UAS3 [−149 to −127]) required for *PIS1* gene expression were identified (25). Consistent with our results, potential Ste12p and Pho2p binding sites were identified in *PIS1* UAS1, while *PIS1* UAS2 contains an additional potential Pho2p binding site.

These results prompted us to quantify *PIS1-lacZ* expression in *pho2*Δ, *yfl044c*Δ, and *ste12*Δ mutant strains. *PIS1-lacZ* expression generally decreased in the three mutant strains, but the effect was most obvious in the *pho2*Δ and *yfl044c*Δ mutants (Fig. 5). The homeodomain transcription factor Pho2 is known to interact with Swi5, Pho4, and Bas1 in vivo and activate transcription of the *HO* gene (7, 9), phosphate utilization genes (7, 82), and genes involved in purine and histidine biosynthesis (7), respectively. Because Pho2 associates with these three proteins, we also quantified *PIS1-lacZ* expression in *swi5*Δ, *pho4*Δ, and *bas1*Δ mutant strains. *PIS1-lacZ* expression increased 34% in the *bas1*Δ strain, while *pho4*Δ and *swi5*Δ mutant strains exhibited lower levels of *PIS1-lacZ* expression (53% and 34%, respectively) relative to that of the wild-type strain (Fig. 5). Because Pho4 and Pho2 are required for induction of target genes in response to low phosphate concentrations (5, 39, 40, 43, 51, 67, 68), we also quantified the effect of phosphate on *PIS1-lacZ* expression. However, we found that *PIS1-lacZ* expression was not significantly affected by phosphate concentration (data not shown).

The *ste12*Δ mutant did not dramatically affect *PIS1-lacZ* expression under the condition we tested or in response to pheromone induction (data not shown). However, Ste12 may affect *PIS1* expression under conditions we have not yet tested since Ste12 is known to regulate a diverse set of genes in response to at least two different signals (16, 35, 62, 83).

The effect of the *yfl044c*Δ mutant on *PIS1-lacZ* expression is the first evidence of regulation of gene expression by this putative transcription factor. While the role of Yfl044c was defined by the ChIP-on-chip database, the only other evidence to suggest that it regulates *PIS1* expression was our β-galactosidase liquid assay. To corroborate the role of Yfl044c in *PIS1* expression, we transformed a pRS200 derivative containing the

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) (Fig. 6A) (25). Deletion of UAS1 decreased *PIS1-cat* expression by half (Fig. 6A, compare pPIS-224 and pPIS-205), while deletion of UAS2 exacerbated the decrease in *PIS1-cat* expression by another 90% (Fig. 6A, compare pPIS-205 and pPIS-149). *PIS1-cat* 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 (URS$_{GLY}$), 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* URS$_{GLY}$ function. The 25-bp region (−76 to −51) defined by the deletion analysis was inserted into plasmid pLGΔ312, previously utilized to test URS function (32, 49). Plasmid pLGΔ312 contains the *CYC1* promoter fused to the *lacZ* reporter gene. The 25-bp *PIS1* URS$_{GLY}$ caused *CYC1-lacZ* expression to decrease dramatically (Fig. 6B). This result indicates that the *PIS1* URS$_{GLY}$ sequence functions as a URS element. The URS$_{GLY}$ 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
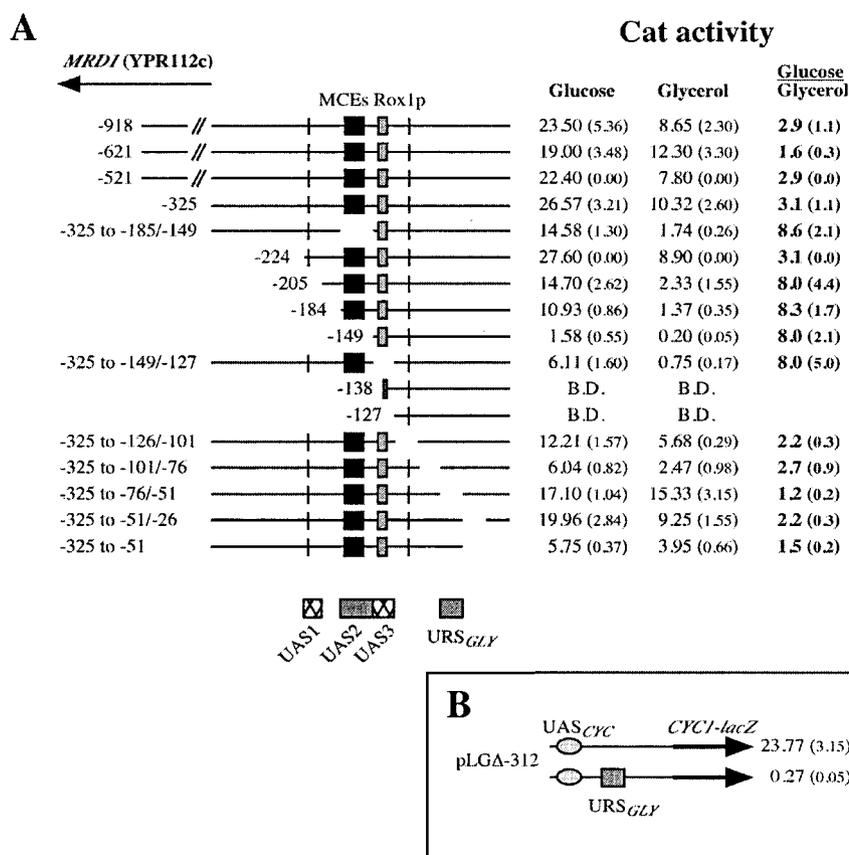
A

MRD1 (YPR112c)	Glucose	Glycerol	Glucose/Glycerol
-918	23.50 (5.36)	8.65 (2.30)	2.9 (1.1)
-621	19.00 (3.48)	12.30 (3.30)	1.6 (0.3)
-521	22.40 (0.00)	7.80 (0.00)	2.9 (0.0)
-325	26.57 (3.21)	10.32 (2.60)	3.1 (1.1)
-325 to -185/-149	14.58 (1.30)	1.74 (0.26)	8.6 (2.1)
-224	27.60 (0.00)	8.90 (0.00)	3.1 (0.0)
-205	14.70 (2.62)	2.33 (1.55)	8.0 (4.4)
-184	10.93 (0.86)	1.37 (0.35)	8.3 (1.7)
-149	1.58 (0.55)	0.20 (0.05)	8.0 (2.1)
-325 to -149/-127	6.11 (1.60)	0.75 (0.17)	8.0 (5.0)
-138	B.D.	B.D.	
-127	B.D.	B.D.	
-325 to -126/-101	12.21 (1.57)	5.68 (0.29)	2.2 (0.3)
-325 to -101/-76	6.04 (0.82)	2.47 (0.98)	2.7 (0.9)
-325 to -76/-51	17.10 (1.04)	15.33 (3.15)	1.2 (0.2)
-325 to -51/-26	19.96 (2.84)	9.25 (1.55)	2.2 (0.3)
-325 to -51	5.75 (0.37)	3.95 (0.66)	1.5 (0.2)

UAS1 UAS2 UAS3 URS$_{GLY}$

B

pLGΔ-312 UAS$_{CYC}$ CYC1-lacZ → 23.77 (3.15)
URS$_{GLY}$ → 0.27 (0.05)

FIG. 6. A. Identification of a *PIS1* promoter element required for glycerol-mediated repression. BRS1001 (wild-type) transformants containing various promoter deletions were grown in synthetic medium lacking uracil and containing either 2% glucose or 3% glycerol. Yeast extracts from the transformants were prepared and assayed for CAT activity. *MRD1* is a divergent ORF. The locations of two MCEs (Mcm1p binding sites) (black box), a Rox1p binding site (gray box), potential TATA boxes (bars), three UAS elements, and a URS element are shown. B.D., below detection. B. *PIS1* URS$_{GLY}$ element functions as a URS element. Plasmid pLGΔ312 + URS$_{GLY}$, containing 25 bp of *PIS1* gene promoter (-76 to -51), was transformed into a wild-type strain (BRS1001), and β-galactosidase activity was measured. Cultures were grown in Ura$^-$ 2% glucose medium.

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 *PIS1-cat* expression (Fig. 7).

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 *PIS1-cat* expression by using the pPIS-325 construct. In addition to glycerol, *PIS1-cat* expression is reduced ~50% when cells are grown with acetate, ethanol, or lactate as a carbon source (Fig. 8).

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, *CHO1-lacZ* expression levels in I$^-$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 I$^+$C$^+$ medium; however, because expression is repressed in this medium, the overall expression levels are lower than those observed in the

I$^-$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 *PIS1* expression. This examination was done by quantifying CAT activity from pPIS1-918 transformants of BRS1001. These experiments revealed that neither growth phase nor hyperosmotic medium affected expression of the *PIS1-cat* gene (Fig. 9).

DISCUSSION

Until recently, *PIS1* gene expression was not believed to be regulated (2, 25, 45, 75, 76). However, we have previously shown that *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 *PIS1* expression. The genomic screen of the VYDS identified 69 mutants with reduced *PIS1* expression in glucose and 51 mutants with increased *PIS1* expression in glycerol. *PIS1* promoter deletion analysis coupled with mining of databases of
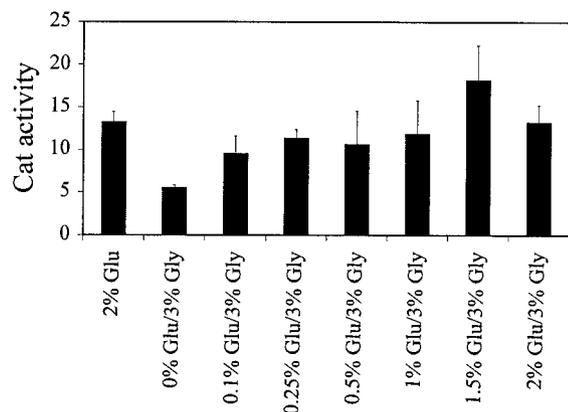
FIG. 7. Glycerol repression of *PIS1-cat* expression is reversed by glucose. BRS1001 transformants containing pPIS-325 were grown in Ura⁻ synthetic medium containing 3% glycerol and 0, 0.1, 0.25, 0.5, 1.0, 1.5, or 2% glucose, and CAT activity was assayed.

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 *PIS1* (25). Comparison of promoters from related *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 *S. cerevisiae* and *S. castellii*, *S. bayanus*, *S. kluyveri*, *S. kudriavzevii*, and *S. mikatae* (25). Mining of the yeast ChIP-on-chip database iden-
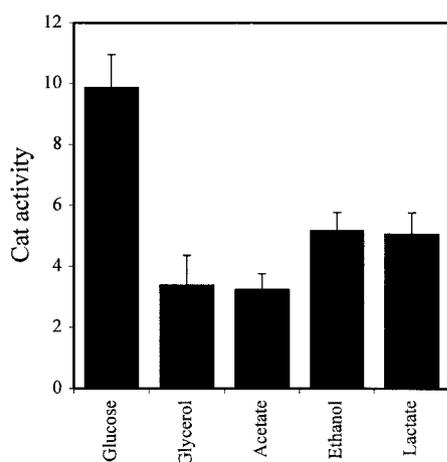

FIG. 9. A. *PIS1-cat* expression is not regulated by growth phase or hyperosmotic stress. BRS1001 transformants containing pPIS1-918 were grown in Ura⁻ synthetic medium in the presence of inositol and choline (I+C+) (solid squares), in the absence of inositol and choline (I−C−) (open squares), in the presence of 0.7 M NaCl (solid circles), or in the presence of inositol, choline, and 0.7 M NaCl (open circles). Yeast extracts from the transformants were prepared and assayed for CAT activity. B. Growth patterns of cultures used as described above (A).

FIG. 8. Analysis of *PIS1-cat* expression in response to different carbon sources. A *PIS1* promoter fragment (pPIS-325) created by PCR was fused to the *cat* reporter gene and integrated in single copy at the *GAL4* locus in strain BRS1001 (wild type). *PIS1-cat* expression (pPIS-325) was assayed in BRS1001 transformants grown in Ura⁻ medium containing 2% glucose, 3% glycerol, 3% acetate, 3% ethanol, or 3% lactate.
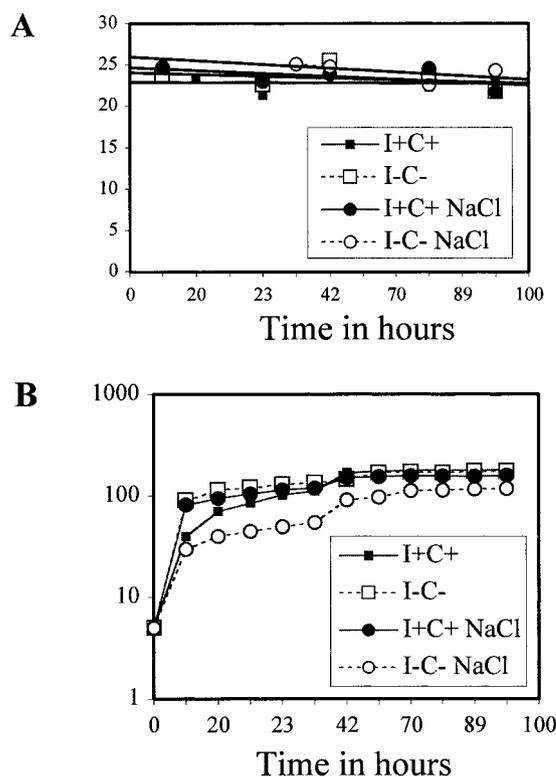
tified Yfl044c and Mcm1 as candidate *PIS1* promoter binding proteins (47).

This study demonstrated that genomic approaches provide an excellent means to identify regulatory mechanisms that control modestly regulated and essential genes such as *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 *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 *PIS1* promoter to exert anaerobic regulation on the *PIS1* gene (25). Conversely, the VYDS screen we employed here did not identify Yfl044c, and examination of the *PIS1* promoter sequence does not reveal an obvious binding site for Yfl044c (TTCTTKTYYTTTT) (47). However, the ChIP-on-chip study placed Yfl004c on the cusp of the acceptable binding threshold (*P* value of 0.006) (47), and we have shown here that Yfl044c does regulate *PIS1-lacZ* expression. The most obvious *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 Grf10), 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 (*acb1* and *gpd1*), chromatin silencing (*rif1*, *hst3*, *hst4*, and *sin3*), and DNA repair (*ptc3*, *ogg1*, *ntg2*, *rpb4*, and *rpb9*). 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, reminiscent of what was seen for *CIT2* in cells with altered mitochondrial activity (10, 13). This finding further supports the existence of a pathway for retrograde regulation between peroxisomes and the nucleus and is suggestive of the existence of a novel mechanism for the cell to adjust to changes in peroxisomal activity.

Note the identification of chromatin silencing and DNA repair mutants by the VYDS screen. The dual roles *HST3* and *HST4* play in chromatin silencing and regulating short-chain fatty acid metabolism again suggest a relationship between *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$_{GLY}$ 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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