Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 350 (2006) 91–96
BBRC
www.elsevier.com/locate/ybbrc

Expression of the *Saccharomyces cerevisiae PIS1* gene is modulated by multiple ATGs in the promoter ☆

Stephanie Iverson, Kevin Sonnemann, Adam Reddick, Virginia McDonough *

Department of Biology, Hope College P.O. Box 9000, Holland, MI 49422-9000, USA

Received 28 August 2006
Available online 15 September 2006

Abstract

The *PIS1* gene encodes a key branchpoint phospholipid biosynthetic enzyme, phosphatidylinositol synthase. The *PIS1* promoter contains the unusual feature of three ATG codons (ATGs1, 2, and 3) in-frame with three stop codons, located just before the authentic start codon (ATG4). Using a $PIS1_{promoter}$-lacZ reporter expression system and site-directed mutagenesis, we investigated the role the "upstream" ATG codons play in modulation of *PIS1* expression. Of the single codon changes, mutation of the first ATG (ATG1) resulted in the largest increase of the reporter gene $PIS1_{promoter}$-lacZ expression. All combinations of altered upstream ATG codons also resulted in greater reporter expression. Reverse transcription-PCR revealed that at least some *PIS1* transcripts include all AUG codons, and their synthesis is probably directed by a second TATA box upstream of the putative TATA box. These results indicate that the multiple upstream AUG codons are present in at least some *PIS1* transcripts and negatively impact *PIS1* expression.

Keywords: *PIS1*; Phosphatidylinositol synthase; Expression regulation

Phosphatidylinositol (PI) is an essential phospholipid in the yeast *Saccharomyces cerevisiae*. PI is synthesized from CDP-diacylglycerol (CDP-DG) and inositol by the enzyme PI synthase, encoded by the gene *PIS1* [1]. The *de novo* synthesis of PI diverges from the synthesis of the other major phospholipids, phosphatidlycholine, phosphatidylserine, and phosphatidylethanolamine, at the common precursor CDP-DG. PI may be subject to modification, including phosphorylation, to generate the important signaling molecules phosphoinositides, and PI serves as a precursor in the synthesis of sphingolipids [2] and glycosylphosphatidylinositol anchors [3].

The PI synthase enzyme has been purified, and its kinetics well characterized [4]. The enzyme itself is not regulated by growth phase [5], serine supplements [6], or many lipids [7]. PI synthase enzyme activity is, however, controlled by the availability of its substrates inositol and CDP-DG. CDP-DG concentrations are governed, in turn, by the level of CDP-DG synthase activity [8] and the competing use of CDP-DG in synthesis of phosphatidylserine. Regulation of the levels of the substrate inositol occurs through both modulation of inositol-1-phosphate synthesis by the *INO1* gene and uptake of inositol from the medium [9].

In *S. cerevisiae*, most phospholipid biosynthetic genes are transcriptionally regulated in response to the presence or absence in the media of the soluble precursors inositol and choline. Genes that exhibit this regulation include *INO1* [9], *CHO1* [10], *CKI1* [11], *EKI1* [12], *PSD1* [13], and *CHO2* and *OPI3* [14]. This regulation is mediated in part by the presence of the UAS_{INO}, a promoter sequence that serves as a binding site for the heterodimer Ino2p/Ino4p activator complex [15]. The negative regulator Opi1p is also required for normal regulation and expression of these genes [16]. Interestingly, even though inositol is a

☆ *Abbreviations:* PI, phosphatidylinositol; CDP-DG, CDP-diacylglycerol; RT-PCR, reverse transcription polymerase chain reaction; ORF, open reading frame.

* Corresponding author. Fax: +1 616 395 7125.
E-mail address: mcdonough@hope.edu (V. McDonough).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.08.196

substrate in the reaction, *PIS1* does not exhibit transcriptional regulation in response to the presence of inositol in the growth medium, in spite of the fact that a potential UAS_{INO} is present in the promoter [17]. However, *PIS1* is not without any transcriptional regulation. Several studies have identified modest changes in transcript levels in response to environmental cues. *PIS1* transcription is modestly activated by galactose and repressed by glycerol in the growth medium as compared to glucose-grown cells [18]. Oxygen levels [19] and cytoplasmic concentrations of zinc [20] are also known to regulate transcription. In an effort to identify additional transcriptional regulators, a genomic screen was used to identify several gene products, including Pho2p, that affected the expression of a *PIS1* promoter-lacZ reporter gene fusion [21]. This study also identified several metabolic pathways that affect *PIS1* expression, including peroxisome generation and function, chromatin silencing, and DNA repair.

An unusual feature of *PIS1* promoter is the presence of three ATG codons that are in-frame with three stop codons located just before what is considered the authentic start codon (Fig. 1). One analysis estimated that only 5% of yeast genes contain more that one ATG before the authentic start [22]. In yeast, the role of multiple ATGs in expression has best been examined in *GCN4*, a transcription factor that activates transcription of biosynthetic genes for amino acids and purines in response to either amino acid or purine starvation (reviewed in [23]). *GCN4* expression is regulated by a translational control mechanism that involves four short open reading frames in the mRNA leader [24]. The translation of the *INO2* gene, which encodes a transcriptional activator of phospholipid biosynthetic genes, is also modulated by the presence of an upstream open reading frame [25]. Given the close proximity of upstream ATG and stop codons to the authentic start codon, it is plausible that *PIS1* expression is also controlled by the presence of these AUGs and stop codons in the mRNA leader.

To examine the possible role of these ATGs in regulating the expression of *PIS1*, we used site-directed mutagenesis to alter their sequences. We found that masking of the most upstream translation start codon resulted in significantly higher expression of a *PIS1-lacZ* reporter gene. Further, all possible combinations of two or more ATG codon changes resulted in still higher expression than single codon changes. Analysis of the 5′-end of *PIS1* transcripts by RT-PCR indicated that at least some transcripts do extend far enough upstream as to contain the upstream AUG codons. These data indicate that control of translation initiation is a contributing factor to the regulation of *PIS1* expression.

Materials and methods

Strains and media. The yeast strain W303-1A (*MATa*, *trp1*, *his3*, *ade2-1*, *leu2-3*, *ura3*) was used in this study. Standard methods for yeast manipulation and media were as described [26]. YNB media are standard synthetic complete medium (0.67% yeast nitrogen base, 2% dextrose, supplemented with appropriate amino acids, uracil and adenine [26]. Yeast strains bearing plamids were grown on YNB lacking uracil (YNB-ura).

Mutagenized plasmids were rescued in *Escherichia coli* strain XL1-Blue (Stratagene). All other DNA manipulation and amplifications were performed using *E. coli* strain DH5α.

Plasmid constructions. A 972 bp fragment of the *PIS1* gene including the promoter and the first four codons of the open reading frame was amplified by PCR from plasmid pPI514 [27] using the oligonucleotides 5′-TAGAGGATCTTGCACGCTGGAGTCC-3′ (pisp1) and 5′-CCACA GAACGTGTTCTGCAGTAACC-3′ (pisp2), synthesized by Integrated DNA Technologies. The fragment was ligated into pCR2.1 (Invitrogen) to generate pCR2.1-PISpro. This manipulation also converted the upstream *Eco*R1 site of the *PIS1* promoter into a *Bam*H1 site. The fragment was removed from pCR2.1PISpro by digestion with *Bam*H1 and *Eco*R1, and placed into pBluescript (Stratagene) at the same sites to create pBS-PISpro. All mutagenesis reactions were performed on this construct.

Site-directed mutagenesis. Relevant ATG sequences were mutated into AAG codons using the QuikChange Site-Directed Mutagenesis kit (Stratagene). All oligonucleotides used in mutagenesis were generated by Integrated DNA Technologies and were purified by polyacrylamide gel electrophoresis. Primers used for mutagenesis are described in Table 1.

To create double mutants (ATG1,2; ATG1,3; ATG2,3), we used previously mutated ATG1 or ATG 2 DNA as template and then used the pair of primers described in Table 1 to introduce the second mutation. For mutagenesis of both the first and second ATG in the ATG1,2,3 construct, mutated ATG3 DNA served as template, and the ATG1,2,3 primers described in Table 1 were used.

After mutagenesis, inserts were sequenced to confirm mutagenesis using the ABI Prism Big Dye Terminator Sequencing Reactions on an ABI 301 Automated Sequencer.

After mutagenesis and sequencing, the reporter gene constructs were created by removing the altered *PIS1* promoters from the pBS-PISpro vectors with *Bam*H1 and *Eco*R1 digestion and ligated into YEp357R [28] at the same sites. This places the first four codons of *PIS1* in-frame with the *lacZ* open reading frame and puts *lacZ* under transcriptional control of the *PIS1* promoter.

β-*Galactosidase assays.* Yeast were grown overnight in YNB-ura medium. One milliliter was transferred to fresh YNB-ura medium and

Fig. 1. The promoter of *PIS1*. The three upstream ATG codons are in solid green italics and are identified as 1,2,or 3. The ATG believed to be the authentic start is labeled "4" and is in green. The three in-frame stop codons that occur between the ATG1,2,3 but before the authentic start codon are in red italics. The possible TATA boxes are in blue and double underlined. The putative UAS_{INO} element is in purple italics. The noted *Eco*R1 site was used to fuse the *PIS1* promoter and first four codons to *lacZ*. The *PIS1* sequence was originally described in [27].

Table 1
Primers used in mutagenesis

Codon mutated	Primer pair used
ATG1	5′-CATAAAAAACAAGAGAGGTGGTATGGTTTATTTGCCGTC-3′
	5′-GACGGCAAATAAACCATACCACCTCTCTTGTTTTTTATG-3
ATG2	5′-CATGAGAGGTGGTAAGGTTTATTTGCCGTCACTGTGG-3′
	5′-CCACAGTGACGGCAAATAAACCTTACCACCTCTCATG-3′
ATG3	5′-GGAGCCTTCAAGTAAAGTAAATAAGAGGGAAAGTGTGATAG-3′
	5′-CTATCACACTTTCCCTCTTATTTACTTTACTTGAAGGCTCC-3′
ATG1, 2, 3	5′-CATAAAAAACAAGAGAGGTGGTAAGGTTTATTTGCCGTC-3′
	5′-GACGGCAAATAAACCTTACCACCTCTCTTGTTTTTTATG-3′

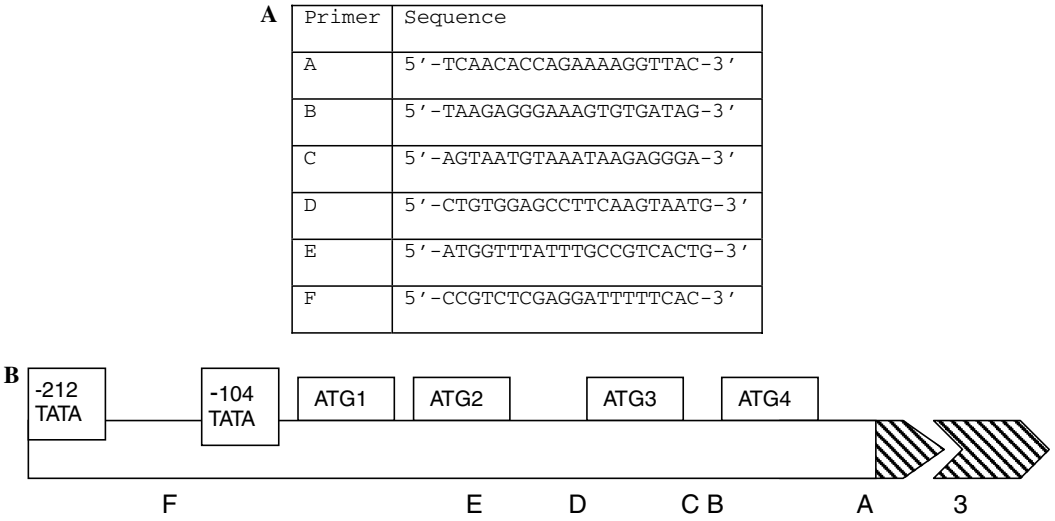

Fig. 2. Upstream primers used in RT-PCR of *PIS1*. (A) Sequence of primers used in RT-PCR. (B) Location of primers used in RT-PCR. The solid bar represents about 215 bases of *PIS1* promoter and the striped bar represents the first 32 bases of coding sequence (not to scale). Primer letter appears below sequence at its relative location. The location of the various ATGs and TATAs is represented above the sequence. Primer 3, used in all RT-PCRs, is found in the middle of the *PIS1* coding sequence.

incubated for four hours at 30 °C. Cells were harvested and β-galactosidase activity was measured using the permeabilized cell method [26]. Enzyme activity was determined using the equation:

$$\beta\text{-Galactosidase activity} = \frac{OD_{420}}{(OD_{600})(\text{Volume of culture tested})(\text{time})}$$

Assays for each construct were performed on three isolates on at least two different days. Constructs were compared to unmodified *PIS1* promoter activity using Student's *t*-test, ($p < 0.05$).

Reverse transcription-PCR (RT-PCR). Total RNA was isolated from W303-1A cells grown to mid log in YNBt/complete medium by the hot phenol method [29]. Contaminating DNA was removed by treatment with DNAse (Promega) following manufacturer's directions. After phenol extraction, first strand cDNA synthesis was performed using Superscript II reverse transcriptase (Gibco BRL, Life Technologies).

To confirm absence of contaminating DNA, actin was used as a control. The primer used for reverse transcription of *ACT1* was 5′-GATC TTCATCAAGTAGTCAGTCAAATCTCTACCGG-3′. PCR amplification on the first strand of actin was performed using two different primers. One primer, posact1up, 5′-GGATTCTGAGGTTGCTTTGGTTATT GATAACG-3′, spans the splice site after intron removal in the mature message and anneals only to mature, spliced message. A primer to the actin intron, inact1up, 5′-CTAGCGCTTGCATCCCATTTAACTGTAA GAAG-3′, was also used. The presence of PCR product with posact1up primer, and the absence of product with inact1up primer confirmed that there was no DNA contamination.

The first strand of *PIS1* was generated by reverse transcription using primer 3, (5′-GTGTAACAATCTGGACTCACC-3′), which is internal to the *PIS1* open reading frame. Then amplification was performed using upstream primers described in Fig. 2. The reactions were denatured on a thermal cycler for 5 min at 94 °C, then cycled 30 times at 94 °C for 45 s, annealed at 50 °C for 45 s, extended at 72 °C for 2 min, and finally extended for 5 min at 72 °C.

Results

Multiple upstream ATGs in the PIS1 gene promoter affect PIS1-lacZ reporter gene activity

The three ATG codons that are in-frame with three stop codons located upstream of what is considered the authentic *PIS1* start codon, ATG4, are shown in Fig. 1. We used site-directed mutagenesis to change each potential start codon from "ATG" to "AAG" separately and then in the indicated combinations. All mutated *PIS1* promoter derivatives were ligated in-frame to the *E. coli lacZ* gene,

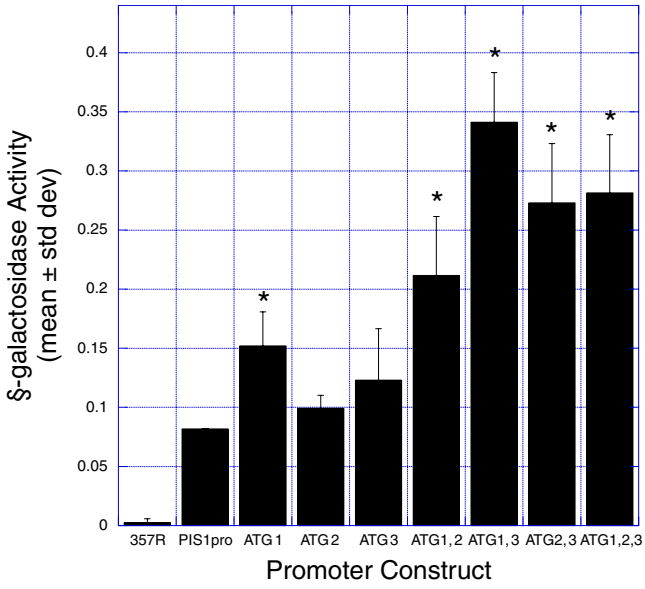

Fig. 3. Reporter gene activity under the control of wild-type and mutated *PIS1* promoters. Expression of *lacZ*, under control of the *PIS1* promoter, was monitored by measurement of β-galactosidase activity as described in Materials and methods. "Yep357" construct has *lacZ* coding sequence, but no *PIS1* promoter, "*PIS1* promoter" is the unmodified *PIS1* promoter, other designations refer to which ATG was mutated in the *PIS1* promoter. An asterisk indicates significant difference from "*PIS1* promoter" construct (Student's *t*-test, $p < 0.05$).

encoding β-galactosidase, for monitoring effects on expression. The effects of the mutations in the *PIS1* promoter-*lacZ* construct on activity of the β-galactosidase are summarized in Fig. 3.

Of the single ATG mutations, changes made to the upstream most sequence (ATG1) resulted in the largest increase in reporter gene expression. ATG2 removal showed minimal effect, that was not significantly different from the unmodified promoter. All double mutations resulted in additional increased activity of reporter gene expression as compared to single mutations. While the single ATG2 mutation had little effect on its own, mutation of it in combination with either ATG1 or ATG3 did result in increased expression over any single mutation. However, the effect of simultaneously mutating ATG1 and ATG3 resulted in a greater effect than either ATG1 or ATG3 in combination with ATG2. This again implies that ATG2 seems to have less effect on expression than ATG1 or ATG3. Finally, the triple mutation resulted in activity roughly equal to the average removal of two upstream start codons.

ATG1 is transcribed in PIS1

The presumed TATA box is located −104 bp before authentic ATG4 [27]. We observed that a mutation in ATG1 has an effect on reporter gene activity. However, ATG1 is located a mere 23 bases from the −104 TATA box. This may not leave much room for efficient transcription and translation of a message starting with ATG1. To investigate whether ATG1 is part of the native *PIS1* message, and therefore likely to influence translation, we performed RT-PCR using probes to various regions of the *PIS1* promoter. Our results indicate that ATG1 is contained in at least some of the *PIS1* transcripts (Fig. 4 lane F).

We did note that there was less RT-PCR product produced with primer C (Fig. 4, lane C) than for other primers flanking each side of C (Fig. 4, compare lane C to lanes B and D). The difference is obvious even though twice as much product for primer C had been loaded on the gel. The reason for this is unknown. It may be due to technical problem with primer C. We do not believe that it represents splicing of the message that would remove the binding site for primer C. First, there is no canonical yeast intron splice signal, TACTAAC [30], found in that region. Second, the messages appear at predicted sizes, although small splices would be hard to detect.

There are two potential TATA boxes, the downstream TATA, which is −104 bp from ATG4, and the upstream TATA, which is −212 bp from ATG4. The evidence

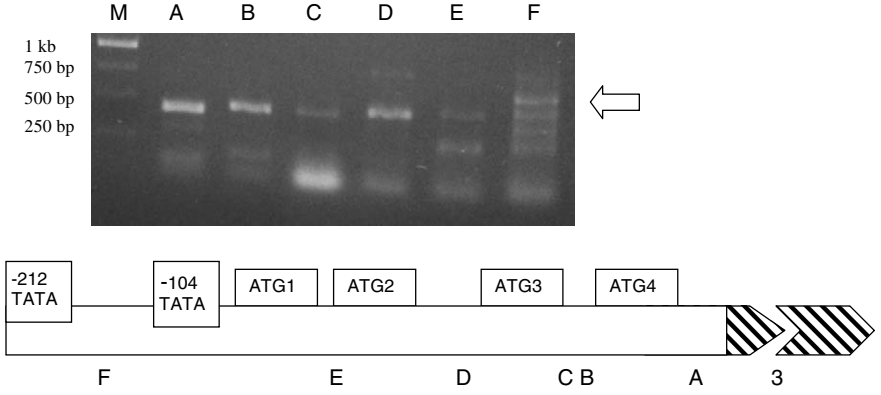

Fig. 4. RT-PCR of *PIS1*. PCR was performed on the first strand of reverse-transcribed *PIS1*, using downstream primer 3 for all, and upstream primer as noted above sample lanes. Five microliters of each PCR product was loaded, except 10 μl of sample C was loaded. The arrow indicates the predicted position of product for primer F and primer 3. Relative locations of primers are indicated in the drawing. "M", 250 bp marker.

provided by RT-PCR demonstrates that there is message that is generated upstream of the −104 TATA box (Fig. 4, lane F). However, there are multiple bands present in this PCR product. We were concerned that the band of interest may be spurious. To confirm that it is an authentic product of the two primers, we ran reactions with either just upstream primer alone (primer F) or both upstream and downstream primers together (primers F and 3). The results demonstrated that the extra bands are due to downstream primer F reaction alone, but that the band of correct size is seen only when we used both upstream and downstream primers simultaneously, indicating it is the authentic product (data not shown).

Discussion

Phosphatidylinositol synthase is a crucial enzyme in the yeast phospholipid biosynthetic pathway. We questioned the role of multiple start and stop codons in the promoter of the *PIS1* gene in the expression of PI synthase. We have observed that altering ATGs 1–3 from the *PIS1* leader sequence causes an increase in expression of a reporter gene construct. This work demonstrates that translational control may play a part in the expression of *PIS1*. The greatest effect was observed when ATG1 was mutated. This is consistent with the primacy of the first encountered AUG to initiate translation [31]. In addition, scanning and use of the first AUG is tempered by context, and ATG1 (and the authentic ATG4) have the best fit to the canonical sequences surrounding AUGs for optimal expression (Fig. 5) [32]. ATG2 appeared to have the least effect on reporter gene expression, in both single and double mutations. This is also in line with lack of good consensus sequences surrounding ATG2.

The *PIS1* TATA box located at −104 bases from ATG4 has been considered the authentic TATA box. This places ATG1 only 23 bases away from this downstream TATA, while ATG2 is 35 and ATG3 is 71 bases away. While some genes have reported to be translated with as little as one base leader [33], it is generally considered that a leader of several bases is necessary for translation. Given that transcription usually occurs several bases downstream of TATA, there may be little leader for AUG1 to be efficiently expressed. In theory then, in order for ATG1 to be efficiently expressed, transcripts may need to begin before the −104 TATA. There is another potential TATA box further upstream at −212. Our RT-PCR results demonstrate that there is at least some message that begins before the −104 TATA, implicating the −212 TATA as a site for transcription start for at least a subset of messages. Transcription from the −212 TATA would offer a leader of sufficient length for efficient transcription and translation of ATG1. The primary *PIS1* transcript has been identified as approximately 1.2 kbp in length [27], and the transcript size appears large relative to the coding sequence range of 660 bp, suggesting that it may include the upstream AUG codons. Additional evidence for the use of both TATAs, besides what we have presented here, can be gleaned from the work of Lopes and colleagues. They created constructs of *PIS1-lacZ* that lack the upstream −212 TATA box, and expression was still observed, implying that the downstream TATA is operational [18]. In that same study however, removal of the upstream TATA did significantly reduce reporter gene activity. It may be that under normal conditions both TATA boxes are functional.

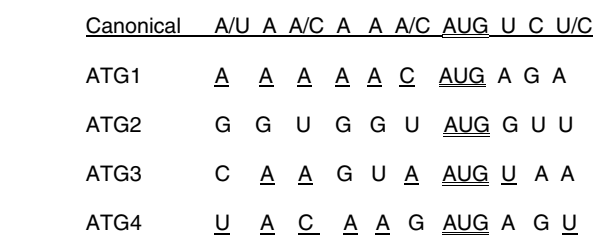

Fig. 5. Context of upstream and authentic PIS1 AUGs. The four AUGs transcribed from the *PIS1* promoter and their flanking sequences are shown. The AUGs are double underlined for emphasis. Neighboring bases that match canonical sequences are underlined.

In later work, Gardocki and Lopes mapped the 5′ end of *PIS1*. Their work indicates that there is a short leader sequence, which only would include ATG3 of the three possible upstream ATGs [19]. However, we observed our largest impact on reporter gene activity when ATG1 was mutated. It is possible that messages generated upstream of ATG3 are in the minority. Our finding of the impact of the removal of ATG1 may reflect the importance of ATG1 in this subset of messages. Alternatively, it is possible that mutation of ATG1 may be altering a binding site for a transcriptional repressor, thereby increasing transcriptional activity, instead of affecting translational efficiency.

In conclusion, we have demonstrated that the *PIS1* promoter modulates expression of a reporter gene by the presence of multiple in-frame start and stop codons in the leader sequence. Future work will be directed at investigating the role these short ORFs play in the normal expression of *PIS1* and whether this modulation of expression by the short ORFs in the leader may help explain the apparent lack of regulation of *PIS1* by inositol.

Acknowledgments

This work was supported by a Hope College-Howard Hughes Medical Institute Faculty Development Grant (to VMM) and by a National Science Foundation Research Experience for Undergraduates Grant BIR #DB1-9820571.

References

[1] J.-I. Nikawa, S. Yamashita, Molecular cloning of the gene encoding CDP diacylglycerol-inositol 3-phosphatidyl transferase in *Saccharomyces cerevisiae*, Eur. J. Biochem. 143 (1984) 251–256.
[2] R.C. Dickson, R.L. Lester, Yeast sphingolipids, Biochim. Biophys. Acta 1426 (1999) 347–357.

[3] S.D. Leidich, D.A. Drapp, P. Orlean, A conditionally lethal yeast mutant blocked at the first step in glycosyl phosphatidylinositol anchor synthesis, J. Biol. Chem. 269 (1994) 10193–10196.
[4] G.M. Carman, J. Matas, Solubilization of microsomal-associated phosphatidylserine synthase and phosphatidylinositol synthase from *Saccharomyces cerevisiae*, Can. J. Microbiol. 27 (1981) 1140–1149.
[5] M.J. Homann, M.A. Poole, P.M. Gaynor, C.T. Ho, G.M. Carman, Effect of growth phase on phospholipid biosynthesis in *Saccharomyces cerevisiae*, J. Bacteriol. 169 (1987) 533–539.
[6] M.J. Homann, A.M. Bailis, S.A. Henry, G.M. Carman, Coordinate regulation of phospholipid biosynthesis by serine in *Saccharomyces cerevisiae*, J. Bacteriol. 169 (1987) 3276–3280.
[7] M. Bae-Lee, G.M. Carman, Regulation of yeast phosphatidylserine synthase and phosphatidylinositol synthase activities by phospholipids in Triton X-100/phospholipid mixed micelles, J. Biol. Chem. 265 (1990) 7221–7226.
[8] H. Shen, W. Dowhan, Regulation of phospholipid biosynthetic enzymes by the level of CDP-diacylglycerol synthase activity, J. Biol. Chem. 272 (1997) 11215–11220.
[9] J. Hirsh, S.A. Henry, Expression of the *Saccharomyces cerevisiae* inositol-1-phosphate synthase (*INO1*) gene is regulated by factors that affect phospholipid synthesis, Molec. Cell. Biol. 6 (1986) 3320–3328.
[10] A.M. Bailis, M.A. Poole, G.M. Carman, S.A. Henry, The membrane-associated enzyme phosphatidylserine synthase is regulated at the level of mRNA abundance, Mol. Cell Biol. 7 (1987) 167–176.
[11] K. Hosaka, T. Murakami, T. Kodaki, J.-C. Nikawa, S. Yamashita, Repression of choline kinase by inositol and choline in *Saccharomyces cerevisiae*, J. Bacteriol. 172 (1990) 2005–2012.
[12] M.C. Kersting, H.-S. Choi, G.M. Carman, Regulation of the yeast *EKI1*-encoded ethanolamine kinase by inositol and choline, J. Biol. Chem. 279 (2004) 35353–35359.
[13] P. Griac, Regulation of yeast phospholipid biosynthetic genes in phosphatidylserine decarboxylase mutants, J. Bacteriol. 179 (1997) 5843–5848.
[14] P.M. Gaynor, T. Gill, S. Toutenhoofd, S.E.F.P. McGraw, M.J. Homann, S.A. Henry, G.M. Carman, Regulation of phosphotidylethanolamine methyltransferase and phospholipid methyltransferase by phospholipid precursors in *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1090 (1991) 326–332.
[15] A.M. Bailis, J.M. Lopes, S.D. Kohlwein, S.A. Henry, *cis* and *trans* regulatory elements required for regulation of the CHO1 gene of *Saccharomyces cerevisiae*, Nucleic Acids Res. 20 (1992) 1411–1418.
[16] L.S. Klig, M.J. Homann, G.M. Carman, S.A. Henry, Coordinate regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae*: pleiotropically constitutive opi1 mutant, J. Bacteriol. 162 (1985) 1135–1141.
[17] A.S. Fischl, M.J. Homann, M.A. Poole, G.M. Carman, Phosphatidylinositol synthase from *Saccharomyces cerevisiae*, reconstitution, characterization, and regulation of activity, J. Biol. Chem. 261 (1986) 3178–3183.
[18] M.S. Anderson, J.L. Lopes, Carbon source regulation of *PIS1* gene expression in *Saccharomyces cerevisiae* involves the *MCM1* gene and the two-component regulatory gene *SLN1*, J. Biol. Chem. 271 (1996) 26596–26601.
[19] M.E. Gardocki, J.M. Lopes, Expression of the yeast *PIS1* gene requires multiple regulatory elements including a Rox1p binding site, J. Biol. Chem. 278 (2003) 38646–38652.
[20] S.-H. Han, G.-S. Han, W.M. Iwanyshyn, G.M. Carman, Regulation of the *PIS1*-encoded phosphatidylinositol synthase in *Saccharomyces cerevisiae* by zinc, J. Biol. Chem. 280 (2005) 29017–29024.
[21] M.E. Gardocki, M. Bakewell, D. Kamath, K. Robinson, K. Borovicka, J.M. Lopes, Genomic analysis of *PIS1* gene expression, Eukaryot. Cell 4 (2005) 604–614.
[22] A.M. Cigan, T.F. Donahue, Sequence and structural features associated with translational initiator regions in yeast—a review, Gene 59 (1987) 1–18.
[23] A.G. Hinnebusch, General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*, in: E.W. Jones, J.R. Pringle, J.R. Broach (Eds.), The Molecular and Cellular Biology of the Yeast Saccharomyces. Gene Expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992, pp. 319–414.
[24] P.P. Mueller, A.G. Hinnebusch, Multiple upstream AUG codons mediate translational control of GCN4, Cell 45 (1986) 201–207.
[25] D.A. Eiznhamer, B.P. Ashburner, J.C. Jackson, K.R. Gardenour, J.M. Lopes, Expression of the *INO2* regulatory gene of *Saccharomyces cerevisiae* is controlled by positive and negative promoter elements and an upstream open reading frame, Mol. Microbiol. 39 (2001) 1395–1405.
[26] M.D. Rose, F. Winston, P. Heiter, Methods in Yeast Genetics: A Laboratory Course Manual, ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1990.
[27] J.-i. Nikawa, T. Kodaki, S. Yamashita, Primary structure and disruption of the phosphatidylinositol synthase gene of *Saccharomyces cerevisiae*, J. Biol. Chem. 262 (1987) 4876–4881.
[28] A.M. Myers, A. Tzagoloff, D.M. Kinney, C.J. Lusty, Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions, Gene 45 (1986) 299–310.
[29] M.E. Schmitt, T.A. Brown, B.L. Trumpower, A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*, Nucleic Acids Res. 18 (1990) 3091–3092.
[30] L.P. Lim, C.B. Burge, A computational analysis of sequence features involved in recognition of short introns, Proc. Natl. Acad. Sci. USA 98 (2001) 11193–11198.
[31] M. Kozak, Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs, Nucleic Acids Res. 12 (1984) 857–872.
[32] H. Miyasaka, The positive relationship between codon usage bias and translation initiation AUG context in *Saccharomyces cerevisiae*, Yeast 15 (1999) 633–637.
[33] D. Najarian, M.E. Dihanich, N.C. Martin, A.K. Hopper, DNA sequence and transcript mapping of *MOD5*: features of the 5′ region which suggest two translational starts, Mol. Cell. Biol. 7 (1987) 185–191.