Combinatorial regulation of phospholipid biosynthetic gene expression by the *UME6*, *SIN3* and *RPD3* genes

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ABSTRACT

The Ume6p–Sin3p–Rpd3p complex negatively regulates expression of genes containing a Ume6p binding site. However, these regulatory proteins also function independently to regulate gene expression both negatively and positively. The model system for this combinatorial regulation is the yeast phospholipid biosynthetic pathway. Sin3p negatively regulates the *INO1*, *CHO1*, *CHO2* and *OPI3* genes while Ume6p negatively regulates the *INO1* gene and positively regulates the other genes. We have suggested that the positive regulation results from indirect effects on expression of the *INO2* transcriptional activator gene. Here, we demonstrate that the effect of Ume6p on *INO2* gene expression is also indirect. We also show that Rpd3p is a negative regulator of phospholipid biosynthetic gene expression. The ability of Ume6p, Sin3p and Rpd3p to differentially regulate expression of the phospholipid biosynthetic genes affects phospholipid composition. A *sin3* mutant strain lacks detectable levels of phosphatidylethanolamine and elevated levels of phosphatidylcholine (PC) and a *rpd3* mutant strain has reduced levels of PC. These alterations in membrane composition suggest that there may exist additional differences in regulation of phospholipid biosynthetic gene expression and that membrane compositions may be coordinated with other biological processes regulated by Ume6p, Sin3p and Rpd3p.

INTRODUCTION

The Ume6p–Sin3p–Rpd3p complex has received considerable attention due to its role in repression of gene expression (1–4). Ume6p interacts with specific DNA sequences found in the promoters of a diverse set of yeast genes (5,6). Sin3p functions as a co-repressor by recruiting Rpd3p to the DNA-bound Ume6p. Rpd3p deacetylates histones H3 and H4, resulting in repression of gene expression (2,3). This model is now well established and the roles of Sin3p and Rpd3p in repression are of particular interest because they are conserved in other

eukaryotes (4). However, it is also well established that these proteins function independently of each other to effect both positive and negative regulation. One of the best examples of this combinatorial control is the phospholipid biosynthetic pathway. The *UME6* and *SIN3* genes are required for both positive and negative regulation of phospholipid biosynthetic gene expression. Here we have examined the roles of the *UME6*, *SIN3* and *RPD3* genes in regulating transcription of the phospholipid biosynthetic genes and the consequent effect on membrane composition.

Transcriptional regulation of phospholipid biosynthesis has been extensively studied in yeast (reviewed in 7,8). Transcription of the *INO1*, *CHO1*, *CHO2* and *OPI3* structural genes is repressed by growth in the presence of inositol and choline and derepressed by growth in their absence. This regulation is dictated by a conserved element present in the respective promoters called the UAS$_{INO}$ element. This element is a binding site for the products of the *INO2* and *INO4* regulatory genes (9,10). Ino2p and Ino4p belong to the basic helix–loop–helix (bHLH) family of proteins, which dimerize through the HLH domains and interact with DNA via the basic regions (11). The precise mechanism for derepression of gene expression has not been elucidated, although it is known that derepression of the *INO2* gene is required (12). The *INO2* gene is autoregulated in response to inositol and choline in a pattern identical to that of its target genes (13). Conversely, repression of the phospholipid biosynthetic genes does not require repression of *INO2* transcription (12). However, it does require the *OPI1* gene, which encodes a leucine zipper protein that is glutamine-rich. Strains deleted for the *OPI1* gene express the phospholipid biosynthetic genes at constitutive derepressed levels (7,8).

Previous studies have suggested that Ume6p and Sin3p can function as both repressors and activators of transcription, in concert or independently. Ume6p binds to a DNA sequence referred to as URS1, which is present in a large number of yeast genes, including the *INO1* phospholipid biosynthetic gene (5,6,14). The *UME6* gene is required for the negative regulation of meiotic genes (15–17), heat shock genes (18), arginine catabolic genes (19) and the *INO1* gene (14). While its ability to repress expression of the meiotic genes and the *INO1* gene is *SIN3*-dependent, the effect on the arginine catabolic genes is *SIN3*-independent. Ume6p can also positively regulate some early meiotic genes during sporulation (20,21), a DNA

repair gene (22), several phospholipid biosynthetic genes (*CHO1*, *CHO2* and *OPI3*) (14) and the *INO2* regulatory gene (14). The ability to function as an activator of early meiotic gene expression is dependent on the *IME1* gene product, which has been shown to interact with Ume6p (23). We have previously suggested that the role of Ume6p on *CHO1*, *CHO2* and *OPI3* gene expression is indirect via regulation of *INO2* gene expression (14). Unlike the situation with the early meiotic genes, the *CHO1* gene is not regulated by the *IME1* gene (14). Consequently, activation of *INO2* expression (and *CHO1* expression) must involve a novel *IME1*-independent mechanism.

Sin3p is a negative regulator of the early meiotic genes (24), the *HO* gene (25), the *TRK2* gene (26) and several phospholipid biosynthetic genes (27). In the case of the *HO* gene, repression does not require the *UME6* gene. In the case of the *CHO1*, *CHO2* and *OPI3* phospholipid biosynthetic genes, repression does not require a URS1 element or the *UME6* gene but does require the UAS$_{INO}$ element (28). While most of the evidence suggests that the *SIN3* gene encodes a negative regulator, *SIN3* is also a positive regulator of the *STE6* gene (29).

The *RPD3* gene is required for repression of *IME2*, *SPO13* and *INO1* transcription (1–3). Moreover, studies have shown that Ume6p specifically recruits the Sin3p–Rpd3p dimer to the URS1 element of the *INO1* promoter, which controls deacetylation of histones H3 and H4. Here we report the role of the *RPD3* gene in regulating phospholipid biosynthesis, which is mediated through the UAS$_{INO}$ element. We also establish a novel pathway for *UME6* positive regulation that involves control of *INO2* transcription. Lastly, we have evaluated the effects of mutations in each of the three regulatory proteins on membrane composition. The mutant phenotypes suggest additional differential control.

MATERIALS AND METHODS

Yeast strains, plasmids and growth conditions

The yeast strains used in this study were: FT5 (MATα, *ura3-52*, *trp1-Δ63*, *his3-Δ200*, *leu2::PET56*) (30), ume6Δ (*ume6::LEU2*), sin3Δ (*sin3::HIS3*) and rpd3Δ (*rpd3::HIS3*), which are isogenic to FT5 (1,30); AMP1008 (MATα, *GAL1-IME1::TRP1*, *gal80::LEU2*, *arg6*, *ura3*, *leu2*, *trp1*, *lys2*, *ho::LYS2*) (20) and the isogenic AMP1170 (*rim16-12*) (15); BRS1001 (MAT**a**, *ade2-1*, *his3-11,15*, *leu2-3-112*, *can1-100*, *trp1-1*, *ura3-1*) (this laboratory); BRS2009 (MAT**a**, *ade2-1*, *his3-11,15*, *leu2-3-112*, *can1-100*, *trp1-1*, *ura3-1*, *ume6::LEU2*) (14); BRS2013 (MAT**a**, *ade2-1*, *his3-11,15*, *leu2-3-112*, *can1-100*, *trp1-1*, *ura3-1*, *ume6::LEU2*, *ura3::pGAL1-INO2::URA3*, *ino2::TRP1*) (this study); BRS1005 (MAT**a**/MATα, *ade1/ade1*, *ino1-13/ino1-13*) (this laboratory). Yeast cultures were grown at 30°C in complete synthetic medium (31) containing 2% glucose (w/v) either supplemented with 75 μM inositol and 1 mM choline (I+C+) or lacking inositol and choline (I–C–). Where indicated, galactose (varying concentrations) and 2% raffinose were used as carbon sources. The X-gal solid medium has been described (27).

Plasmids pTL101, pGAL1-INO2 and pLG669-Z have been described (12,32,33). Plasmid pJH330 contains 543 bp of the sequences upstream of the *INO1* gene and 132 codons of the *INO1* gene fused in-frame to the *lacZ* reporter gene in YEp357R (34). This plasmid was constructed by inserting a 1.0 kb *Pst*I–*Bgl*II fragment containing the *INO1* sequences into the *Pst*I–*Bam*HI sites of YEp357R. Yeast transformations were performed using a routine procedure (35).

Opi plate assay

The Opi test is a cross-feeding assay that assesses the ability of yeast strains to excrete inositol into the growth medium. A modification of the original plate assay was used (36,37).

RNA analyses

Total yeast RNA was isolated using a glass bead disruption and hot phenol extraction procedure (38). Northern blot hybridizations were performed as described previously (39). RNA probes (cRNA) were synthesized using the Riboprobe Combination System SP6/T7 (Promega, Madison, WI) from plasmids (27) linearized with a restriction enzyme as follows (shown as plasmid/restriction enzyme/RNA polymerase) for the indicated (parenthesized) probe: pAB309Δ/*Eco*RI/SP6 (*TCM1*); pAS103/*Hin*dIII/T7 (*CHO1*); pJH310/*Hin*dIII/T7 (*INO1*); pPLg/*Bam*HI/SP6 (*ACT1*). Results were visualized using a phosphorimager and quantitated using ImageQuant from Molecular Dynamics Inc. (Sunnyvale, CA).

β-Galactosidase assays

Yeast transformants were assayed for β-galactosidase activity as previously described (40). Units of β-galactosidase activity are defined as (OD$_{420}$/min/mg total protein) × 1000. Total protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Rockville Center, NY).

Determination of steady-state phospholipid composition

Steady-state labeling of phospholipids with [^{32}P]orthophosphate (NEN DuPont, Boston, MA) was performed essentially as described previously (41). Cells were grown in the presence of [^{32}P]orthophosphate (50 μCi, 9000 Ci/mmol) for at least five generations and harvested at a density of 5×10^7 cells/ml. Labeled cells were suspended in 5 ml of cold 5% trichloroacetic acid and incubated on ice for 20 min. Cells were pelleted and washed twice with distilled H$_2$O and 1 ml of polar solvent (40% ethanol, 13.9% diethyl ether, 2.8% pyridine, 0.027% ammonium hydroxide and 0.01% butylated hydroxytoluene in 2:1 chloroform:methanol) was added. Lipids were extracted by incubation at 60°C for 20 min (42). After cooling to room temperature, a 0.5 vol of distilled H$_2$O and 5 vol of 2:1 chloroform:methanol containing 0.005% butylated hydroxytoluene were added and the mixture was vortexed (43). Solvents were then fractionated by centrifugation (1500 *g*, 10 min) and the lower layer was transferred to a dram vial and dried under a stream of nitrogen gas. The lipid pellet was dissolved in 20 μl of 2:1 chloroform:methanol, spotted onto chromatography paper (Whatman SG81 treated with 2% EDTA, pH 7.4) and separated by 2-dimensional chromatography (44). The solvent for the first dimension was chloroform:methanol:2.8% ammonium hydroxide:dH$_2$O (66:27:3:0.8) and the solvent for the second dimension was chloroform:methanol:acetic acid:dH$_2$O (32:4:5:1). Phospholipids were visualized by autoradiography and quantitated by liquid scintillation.

Electrophoretic mobility shift assay (EMSA)

Recombinant MBP and MBP–Ume6p were generated as described (5). Binding reactions were carried out using three fragments of the *INO2* promoter or a 26 bp oligonucleotide containing a URS1 element from the *SPO13* gene using conditions described previously (5).

Mutagenesis

The *ume6Δ* strain transformed with pTL101 was mutagenized with EMS to 30% survival as described previously (27).

RESULTS

UME6, *SIN3* and *RPD3* repress *INO1* transcription

It is now well established that the URS1 element binds Ume6p, which interacts with Sin3p and Rpd3p to repress target gene expression (1,3). Consistent with this model, we showed previously that the URS1 element in the *INO1* promoter is required for repression of *INO1–lacZ* and *INO1–cat* reporter genes (14,45) and that *UME6* is a negative regulator of *INO1* expression (45). Moreover, a mutant (*cpe1*) defective in repression of an *INO1–lacZ* reporter gene (27) was found to be allelic to *sin3* mutations. These observations suggested that *RPD3* could be a negative regulator of *INO1*.

To test this possibility, we examined *INO1* expression in a *rpd3* mutant strain by northern blot hybridization, by quantitating *INO1–lacZ* expression and by assaying for an Opi⁻ phenotype (46). We had previously observed that a *ume6Δ* mutant strain displays an Opi⁻ phenotype (14). Here, we found that the *sin3Δ* and *rpd3Δ* mutant strains also display an Opi⁻ phenotype (Fig. 1A). Consistent with the Opi⁻ phenotype, repression of both *INO1* transcription (Fig. 1B) and *INO1–lacZ* expression (data not shown) is defective in all three mutant strains. The three assays also show that the defect in *INO1* expression is most pronounced in the *ume6Δ* strain and least pronounced in the *rpd3Δ* strain. This graded defect in repression has been observed by others using a heterologous reporter system that contained either the *INO1* or the *IME2* URS1 elements (1).

UME6, *SIN3* and *RPD3* regulation through the *CHO1* UAS$_{INO}$ element

We had previously shown that *SIN3* is a negative regulator of the *INO1*, *CHO1*, *CHO2* and *OPI3* phospholipid biosynthetic genes in spite of the fact that only the *INO1* promoter contains the URS1 element (27). Later, it was shown that *SIN3* functioned through two UAS$_{INO}$ elements present in the *INO1* promoter (28). The interaction between Sin3p and Ume6p prompted an examination of phospholipid gene expression in a *ume6Δ* strain. Unexpectedly, the *UME6* gene was found to be a positive regulator of *CHO1*, *CHO2* and *OPI3* and a negative regulator of *INO1* (14).

Since the three genes that were positively regulated by the *UME6* gene all contain the UAS$_{INO}$ element, we assayed the effect of a *ume6Δ* mutation on expression of a *lacZ* reporter gene driven by the UAS$_{INO}$ element from the *CHO1* promoter (pTL101) (32). The pTL101 plasmid contains the *lacZ* gene driven by a *CYC1* promoter lacking the *CYC1* UAS element
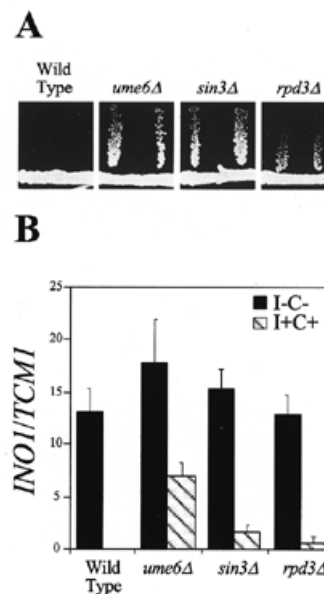

Figure 1. Repression of *INO1* transcription is defective in *ume6Δ*, *sin3Δ* and *rpd3Δ* mutant strains. (**A**) Opi test. Mutants (horizontal) are streaked on 1.2% agar medium lacking inositol and allowed to grow for 72 h. A tester strain (inositol auxotroph, *ino1*) is streaked away (vertical) from the original streak and allowed to grow for 72 h (37). (**B**) *INO1* transcription quantitated by northern blot hybridization and normalized using *TCM1*. I–C–, derepressing medium lacking inositol and choline (solid); I+C+, repressing medium containing 75 μM inositol and 1 mM choline (hatched).
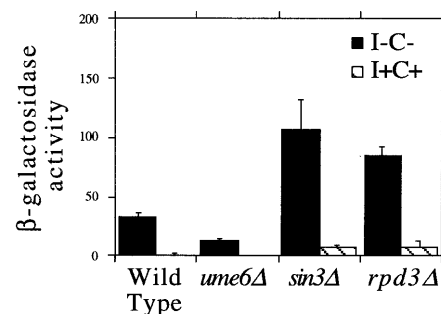

Figure 2. Ume6p functions as a positive regulator and Sin3p and Rpd3p function as negative regulators through the *CHO1* UAS$_{INO}$ element. β-Galactosidase activity from transformants containing pTL101 (32). I–C–, solid; I+C+, hatched.

but containing the *CHO1* UAS$_{INO}$ element (inserted using a 28 bp oligonucleotide).

The data demonstrate that the positive role of *UME6* on *CHO1* expression is exerted through the UAS$_{INO}$ element, since *lacZ* expression was decreased in the *ume6Δ* strain (Fig. 2). This pattern of expression mirrors that of the native *CHO1* gene in that *CHO1* transcription was also decreased in the *ume6Δ* strain (Fig. 3; 14). However, *CHO1* expression in the *ume6Δ* strain was previously shown to be unresponsive to the presence of inositol and choline while *lacZ* expression from pTL101 was still sensitive to inositol and choline (Fig. 2). This could be due to differences in the sensitivities of northern blot
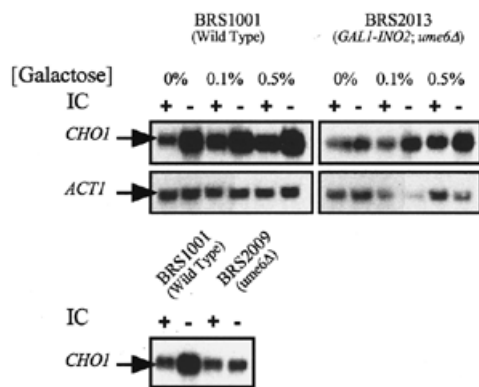
Figure 3. Overexpression of *INO2* rescues a *ume6Δ* phenotype (absence of *CHO1* derepression). Northern blot showing *CHO1* transcript levels in a wild-type strain (BRS1001) and an isogenic *ume6Δ*, *ino2Δ* strain containing a *GAL1* promoter–*INO2* fusion. Northerns were probed with *CHO1*- and *ACT1*-specific cRNA probes. The lower panel shows a control experiment demonstrating the *ume6Δ* phenotype (14). Lanes marked + indicate RNA purified from cells grown in medium containing 75 μM inositol and 1 mM choline while the lanes marked – contain RNA from cells grown in the absence of these precursors. The RNA samples used in the upper panel northerns were grown in medium containing various concentrations of galactose plus 2% raffinose. The RNA samples used in the lower panel northern were grown in medium containing glucose.

hybridizations and β-galactosidase assays. Alternatively, the decreased activity of the UAS$_{INO}$ element in the *ume6Δ* mutant strain may abate the response to inositol and choline when the UAS$_{INO}$ element is in its native context. This possibility is supported by the observation that *lacZ* expression is regulated 18-fold by the *CHO1* UAS$_{INO}$ element in pTL101 while *CHO1* and *CHO1*–*lacZ* expression is regulated 3–4-fold (14,31,47). The data further show that *SIN3* is a negative regulator through the *CHO1* UAS$_{INO}$, as it is through the two UAS$_{INO}$ elements in the *INO1* promoter (28). In addition, *RPD3* was found to function as a negative regulator through the *CHO1* UAS$_{INO}$ element. Therefore, this data identifies two new mechanisms for the action of Ume6p, Sin3p and Rpd3p in addition to the URS1-mediated repression mechanism: Sin3p–Rpd3p-mediated negative regulation through the UAS$_{INO}$ element; and Ume6p-mediated positive regulation through the UAS$_{INO}$ element.

INO2 overexpression restores regulation of *CHO1* expression in a *ume6Δ* strain

Because *UME6* functions as a positive regulator of UAS$_{INO}$-containing genes, we previously suggested that Ume6p either directly interacted with the UAS$_{INO}$ element or that it regulated expression of the genes encoding the UAS$_{INO}$-binding proteins Ino2p and Ino4p. We have shown that *INO2*–*cat* expression is decreased in the *ume6Δ* strain but *INO4*–*cat* expression is unaffected (14).

Therefore, the decreased inositol/choline response of the *CHO1* gene in a *ume6Δ* strain could be due to decreased *INO2* expression. To test this, we examined expression of the *CHO1* gene in a *ume6Δ*, *ino2Δ* strain containing the *INO2* gene driven by the *GAL1* promoter (12). We have previously used this *GAL1*–*INO2* construct, which expresses *INO2* in a galactose-dependent but inositol/choline-independent manner (12). Here

we used it to determine if overexpression of the *INO2* gene restores regulation of *CHO1* gene expression in a *ume6Δ* strain. The data show that *CHO1* expression increased as a function of galactose concentration (Fig. 3; BRS2013). Moreover, regulation of *CHO1* expression in response to inositol and choline was more evident at higher galactose concentrations when *INO2* expression was increased. Galactose did not affect *CHO1* expression in a strain containing a wild-type *INO2* gene (Fig. 3; BRS1001)

To further test if *INO2* overexpression rescues the *CHO1* phenotype, we devised a screen for mutants that express wild-type (or greater) levels of *lacZ* from the pTL101 plasmid in a *ume6Δ* mutant strain. The goal of this screen was to identify mutants that overexpress the *lacZ* reporter, possibly as a result of increased *INO2* transcription. The biggest problem that we anticipated was the isolation of bypass suppressors that would increase *lacZ* expression from the reporter without increasing *INO2* expression. Optimally, we would want to use a reporter system driven by the *INO2* promoter. Unfortunately, the *INO2* promoter is too weak to drive expression of the *lacZ* reporter gene (unpublished observation).

As described above, in a *ume6Δ* strain *lacZ* expression from pTL101 is 70% lower than in the isogenic wild-type strain (I–C– medium) (Fig. 2). Fortunately, this 3-fold difference in β-galactosidase activity is easily observed by plate assay. A wild-type strain turns blue on X-gal I–C– medium after a 24 h incubation whereas the *ume6Δ* strain requires 4 days to turn light blue. This enormous difference in plate phenotype is presumably due to the fact that the β-galactosidase activity generated by the *ume6Δ* strain is at the lower limit of sensitivity of the plate assay.

We treated the *ume6Δ* strain containing pTL101 with EMS and screened for colonies that turned blue after 24 h on X-gal I–C– medium. As a control, mock-mutagenized cells were plated and found to turn light blue after 4 days. Candidate mutant colonies were assayed for β-galactosidase activity by a liquid assay which revealed that those that had turned blue after 24 h yielded wild-type (or greater) levels of activity (Fig. 4A). Northern blot hybridization was performed to identify mutants that overexpressed the *INO2* transcript. Among the original 11 mutant strains, five were found to express wild-type (or greater) levels of the *INO2* transcript (data not shown). All five mutants are recessive and have been designated rescuers of *ume6Δ* (*rum*). All five mutant strains were grown on 5'-FOA plates to select for colonies lacking the pTL101 reporter plasmid and re-transformed with pTL101 to ensure that the phenotype was not plasmid borne. We also examined expression of a *CYC1* promoter-driven *lacZ* reporter (pLG669-Z) in the *rum* mutant strains to determine if any of the *rum* mutants identify general transcription factors. The data show that some of the *rum* mutants (*rum6*, *rum11* and *rum18*) display an increase in expression of the *lacZ* gene relative to the *ume6Δ* parental strain (Fig. 4B). However, the increase is modest relative to the pTL101 phenotype (Fig. 4A) (1.5-fold for the pLG669-Z reporter versus 3.5–6.5-fold for the pTL101 reporter). Moreover, the *rum4* and *rum14* mutants were indistinguishable from the *ume6Δ* parental strain when assayed for the pLG669-Z reporter. We have also determined that the *rum* mutants are not complemented by the *OPI1*, *SIN3* and *RPD3* genes, when they are present on a centromeric plasmid (data not shown).
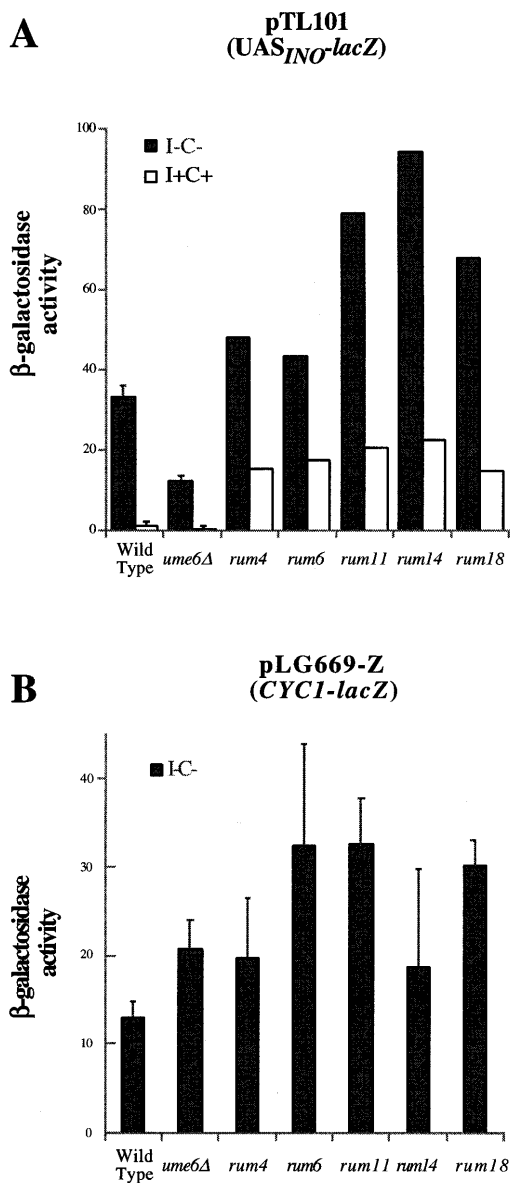
A

pTL101
(UAS$_{INO}$-lacZ)

B

pLG669-Z
(*CYC1-lacZ*)

Figure 4. The *rum* mutations rescue the defect in *lacZ* expression from pTL101 caused by the *ume6Δ* mutant. (**A**) β-Galactosidase activity from transformants containing pTL101 (32). I–C–, solid; I+C+, open. (**B**) β-Galactosidase activity from transformants containing pLG669-Z (33)

UME6 regulates *INO2* gene expression indirectly

The data above shows that *UME6* is a positive regulator of *INO2*. This is not unreasonable given that Ume6p is known to function as both a repressor and activator of transcription (20). However, several conditions are required for Ume6p activator function (20), namely: (i) it functions as an activator in meiotic cells; (ii) it requires the URS1 element; (iii) it requires the *IME1* gene. None of these conditions are met in our experiments with the *INO2* gene (14).

Consequently, Ume6p might regulate *INO2* expression via an indirect mechanism that does not require its activator function. To test this we examined *CHO1* transcription in a
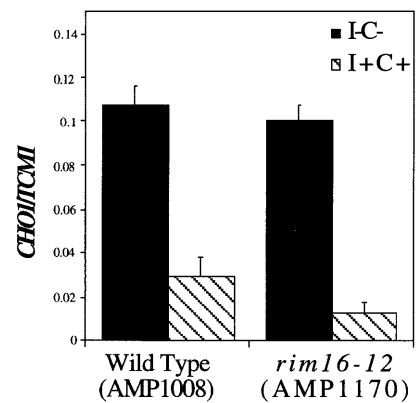

Figure 5. The Ume6p transcriptional activation activity is not required for *CHO1* expression. Quantitative northern blot hybridization using a *CHO1*-specific cRNA probe normalized using a *TCM1*-specific cRNA probe. I–C–, solid; I+C+, hatched.

rim16-12 mutant strain (AMP1170). This allele has a mutation in the *UME6* gene that eliminates activator function but does not affect repressor function (20). The data show that *CHO1* expression in the *rim16-12* strain was indistinguishable from that of the isogenic wild-type strain (Fig. 5). The response to inositol and choline was also observed in the *rim16-12* strain. These data suggest that either *UME6* is regulating *INO2* expression indirectly or directly by a novel *IME1*-independent activation mechanism.

To test if Ume6p directly interacts with the promoter of the *INO2* gene, we performed EMSA using recombinant MBP–Ume6p. We were unable to detect binding of the recombinant MBP–Ume6p to three different fragments of the *INO2* promoter (Fig. 6) even upon prolonged exposure (five times longer than the exposure shown in Fig. 6). The three overlapping fragments used in this study encompass 500 bp of the *INO2* promoter that are responsive to mutations in the *UME6* gene (14). However, the recombinant protein was fully competent for complex formation with a binding site from the *SPO13* gene (Fig. 6).

Mutations in the *UME6*, *SIN3* and *RPD3* genes yield altered membrane phospholipid compositions

Membrane phospholipid composition can be altered by different growth conditions and by mutations in genes that regulate phospholipid biosynthetic gene expression (reviewed in 7,8). One of the more dramatic effects is seen in strains harboring mutations in the *OPI1* negative regulatory gene. The membranes of wild-type strains typically contain 28–30% phosphatidylinositol (PI) when grown in medium containing inositol and 10–12% PI when grown in medium lacking inositol. The is due to the fact that the enzyme which synthesizes PI (PI synthase) is limited for inositol when cells are grown in medium lacking inositol (48). Strains harboring mutant *opi1* alleles contain elevated levels of PI when cells are grown in medium lacking inositol. This effect is due to overexpression of the *INO1* gene that is required for *de novo* synthesis of inositol (7,8).
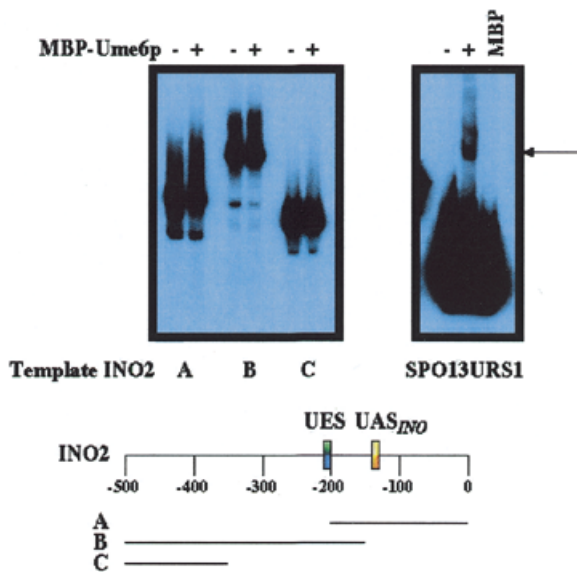
Figure 6. Recombinant MBP–Ume6p does not form complexes with restriction fragments from the *INO2* promoter. Three different restriction fragments from the *INO2* promoter were combined with recombinant MBP–Ume6p. The regions covered by each of the restriction fragments are shown relative to the 500 bp immediately upstream of the *INO2* start codon and UES and UAS$_{INO}$ elements (B.P.Ashburner, D.A.Eiznhamer and J.M.Lopes, unpublished data). As a control, an oligonucleotide containing the URS1 element from the *SPO13* gene was used in reactions with either MBP or MBP–Ume6p.

Because the *ume6*, *sin3* and *rpd3* mutant strains display the Opi⁻ phenotype and because they differentially affect expression of the phosphatidylcholine (PC) biosynthetic genes, we examined the effects of these mutant alleles on phospholipid composition. There are three salient changes in the phospholipid compositions of these strains. The first is an increase in the amount of PI in cells grown in the absence of inositol (Table 1). This phenotype is not unexpected since the *opi1* mutant strains have a similar phenotype (7,8). Interestingly, the effect of the mutant strains on PI composition (Table 1) is graded, as is the effect on *INO1* expression (Fig. 1). Another phenotype is the reduced level of PC in the *rpd3* mutant strain. This reduction in PC was observed in cells grown in the

presence and absence of inositol (Table 1). A third phenotype is the complete absence of phosphatidylethanolamine (PE) in the *sin3* mutant strain. Again, this phenotype was observed regardless of the presence or absence of inositol (Table 1). The absence of PE may account for the increase in PC or it may be due to decreased synthesis of PE or a combination of the two events. Regardless of the explanation for this phenotype, this is the first observation of a mutant that specifically affects PE levels.

DISCUSSION

The role of Ume6p, Sin3p and Rpd3p in regulating phospholipid biosynthesis was examined. It had been previously demonstrated that *UME6* and *SIN3* are required for repression of the *INO1* gene and that this repression requires the URS1 element (1,3,14,27,45). Data presented here demonstrate that *RPD3* is also required for repression of the *INO1* gene (Fig. 1). Curiously, the most striking phenotype of a *rpd3* mutant strain is the Opi⁻ phenotype, which usually correlates with *INO1* overexpression (46). Although both the *INO1* transcript levels and *INO1–lacZ* activity are increased in the *rpd3* mutant strain, the degree of overexpression is modest relative to the Opi⁻ phenotype. Consequently, the discrepancy between the Opi⁻ phenotype and the defect in *INO1* transcription suggests that the *rpd3* mutant may affect some other genes involved in inositol production or excretion.

The data also reveal that Rpd3p represses transcription through the UAS$_{INO}$ element (Fig. 2), which is present in most of the phospholipid biosynthetic genes (7,8). This observation is consistent with the previous finding that Sin3p represses transcription through the UAS$_{INO}$ element (28). At present it is not possible to determine if the effect of Sin3p and Rpd3p through the UAS$_{INO}$ element is direct or indirect, although there is no evidence to support the model of direct binding to the UAS$_{INO}$ element. One possibility is that Sin3p may tether Rpd3 to proteins already bound at the UAS$_{INO}$ element, such as Ino2p and Ino4p. Curiously, the mammalian Mad and Mxi1 proteins interact with the mammalian Sin3p homolog (49,50). Mad, Mxi1, Ino2p and Ino4p are all members of the basic helix–loop–helix family of proteins (7,8,11). Another possibility is that the effect may be indirect by regulating *INO2*

Table 1. Phospholipid composition of *ume6*Δ, *sin3*Δ and *rpd3*Δ mutant strains

Strains	Medium	PI[a]	PS[a]	PE[a]	PC[a]	PA[a]
Wild-type	I–C–	12.5 ± 1.3	9.5 ± 1.2	6.0 ± 2.3	51.1 ± 7.5	2.3 ± 0.6
*ume6*Δ	I–C–	17.8 ± 1.5	10.6 ± 2.1	7.0 ± 2.0	50.7 ± 4.0	1.6 ± 0.1
*sin3*Δ	I–C–	15.7 ± 3.2	12.5 ± 2.5	ND[b]	56.3 ± 2.5	1.1 ± 0.0
*rpd3*Δ	I–C–	13.3 ± 2.1	15.0 ± 2.6	10.0 ± 1.0	43.7 ± 1.2	2.0 ± 0.0
Wild-type	I+C+	32.3 ± 5.1	7.9 ± 1.7	6.4 ± 1.0	41.8 ± 2.6	1.0 ± 0.2
*ume6*Δ	I+C+	28.3 ± 4.1	7.8 ± 0.6	8.6 ± 1.0	46.1 ± 1.4	0.8 ± 0.2
*sin3*Δ	I+C+	27.7 ± 1.5	8.0 ± 1.0	ND	55.3 ± 2.9	0.9 ± 0.1
*rpd3*Δ	I+C+	36.8 ± 9.4	7.0 ± 0.0	4.0 ± 2.6	31.7 ± 9.0	0.9 ± 0.2

[a]PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid.
[b]ND, not detectable.

transcription. However, if this were the case the effect might be redundant since the *INO2* promoter also contains a UAS_{INO} element (D.A.Eiznhamer and J.M.Lopes, unpublished data). Nevertheless, the ability of Sin3p to repress transcription in the absence of Ume6p has been previously documented for the *HO* gene (25). The UAS_{INO}-*lacZ* reporter (Fig. 2) should be a useful tool in genetic screens to define the precise mechanism for Sin3p–Rpd3p repression of phospholipid biosynthetic gene expression.

We previously reported that *UME6* is a positive regulator of phospholipid biosynthetic gene expression (14). Here we show that the positive regulation is exerted through the UAS_{INO} element (Fig. 2). We have also shown that this regulation is indirectly mediated by controlling the amount of *INO2* expression. Overexpression of the *INO2* gene using the *GAL1* promoter (Fig. 3) or in the *rum* mutant strains (Fig. 4) rescues the *CHO1* mutant phenotype. While Ume6p can function as a positive regulator, it usually requires an interaction with Ime1p, which provides the transcriptional activation domain (23). However, the *IME1* gene is not required for proper regulation of *CHO1* transcription (14). This has led us to consider two models for Ume6p positive regulation. One model predicts that Ume6p directly interacts with the *INO2* promoter and associates with a novel protein to activate *INO2* transcription. However, we have not been able to detect binding of Ume6p to the *INO2* promoter (Fig. 6). Moreover, a comprehensive deletion analysis of the *INO2* promoter has identified only two positive *cis*-acting sequences; a TATA-like element and a UAS_{INO} element (D.A.Eiznhamer and J.M.Lopes, unpublished data). Deletion of these elements from the *INO2* promoter completely eliminates *INO2* transcription, which is a very different phenotype from that observed in *ume6* mutant strains. Furthermore, neither of these two positive *cis*-acting sites contain sequences which conform to the Ume6p binding site (51). Thus, we favor a second model that predicts an indirect effect of Ume6p on *INO2* transcription through an intermediate positive regulatory gene. This intermediate gene may be present among the *rum* mutants described in this manuscript. Cloning of the *RUM* gene(s) coupled with mobility shift assays using the *INO2* promoter and extracts from *rum* mutant strains will help distinguish between the models. Furthermore, we have isolated a larger collection of *rum* mutants using an *INO2* promoter–*HIS3* reporter which will also help define the *UME6*-mediated regulation of *INO2* gene expression.

It is a little more difficult to reconcile the effect of the *ume6* mutant on *INO1* expression. This promoter contains both the URS1 element and two UAS_{INO} elements (45,52). Consequently, the *ume6* mutant should result in increased expression through the URS1 element and decreased expression through the UAS_{INO} element. The fact that only the former phenotype is observed suggests that the URS1-mediated regulation is epistatic to UAS_{INO}-mediated regulation. Alternatively, the lowered amount of Ino2p in the *ume6Δ* mutant may be sequestered to the UAS_{INO} elements of the *INO1* promoter. In support of this, it is known that the UAS_{INO} elements of the *INO1* promoter are able to drive significantly greater levels of *lacZ* expression relative to the UAS_{INO} element from the *CHO1* promoter (40; Fig. 2).

The consequence of mutations in the three regulatory genes on phospholipid composition had not been previously examined. The *rpd3* and *sin3* mutants displayed novel profiles. The *rpd3* mutant yielded a substantial decrease in the level of PC (Table 1). This suggests that *rpd3* may affect expression of the *CHO2* or *OPI3* genes in a manner different from its effect on the *CHO1* gene. Alternatively, the *rpd3* mutant may affect expression of genes in the salvage pathway or the PC turnover pathway (7,8). One of the more striking phenotypes is the complete absence of PE in the *sin3* mutant strain (Table 1). It has been demonstrated that *sin3* mutant strains overexpress the *CHO1*, *CHO2* and *OPI3* genes (27). One possible explanation for the phenotype is that overexpression of the *CHO2* and *OPI3* genes depletes the cell of PE at the expense of PC. Another possibility is that the *PSD1* and *PSD2* genes, which convert PS to PE, are down-regulated. These two genes had not been cloned when the *sin3* effect on *CHO1*, *CHO2* and *OPI3* transcription was tested (7,27). Regardless of the mechanism, the finding of a condition where PE is completely absent from the membrane is interesting and raises the possibility that PE may be required for certain biological processes. Interestingly, Mcm1p and Sin3p function as positive regulators in the mating response pathway (29) and the *PSD1* and *PSD2* promoters contain Mcm1p binding sites (J.M.Lopes, unpublished observation). It remains to be determined if PE levels play a role in biological processes such as mating.

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