 Nucleic Acids Research, 1999, Vol. 27, No. 9 **2043–2050**

The yeast inositol-sensitive upstream activating sequence, UAS_{INO}, responds to nitrogen availability

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Received December 10, 1998; Revised and Accepted March 4, 1999

ABSTRACT

The *INO1* gene of yeast is expressed in logarithmically growing, wild-type cells when inositol is absent from the medium. However, the *INO1* gene is repressed when inositol is present during logarithmic growth and it is also repressed as cells enter stationary phase whether inositol is present or not. In this report, we demonstrate that transient nitrogen limitation also causes *INO1* repression. The repression of *INO1* in response to nitrogen limitation shares many features in common with repression in response to the presence of inositol. Specifically, the response to nitrogen limitation is dependent upon the presence of a functional *OPI1* gene product, it requires ongoing phosphatidylcholine biosynthesis and it is mediated by the repeated element, UAS_{INO}, found in the promoter of *INO1* and other co-regulated genes of phospholipid biosynthesis. Thus, we propose that repression of *INO1* in response to inositol and in response to nitrogen limitation occurs via a common mechanism that is sensitive to the status of ongoing phospholipid metabolism.

INTRODUCTION

Structural genes encoding a number of phospholipid biosynthetic enzymes in the yeast *Saccharomyces cerevisiae* show complex transcriptional regulation in response to the availability of the phospholipid precursors, inositol and choline (reviewed in 1–3). The most highly regulated of these genes is *INO1*, the structural gene for inositol-1-phosphate (I-1-P) synthase (4,5). However, a large number of genes encoding other enzymes of phospholipid biosynthesis show similar regulation (reviewed in 1–3,6,7). The basic pattern of regulation is as follows. During the logarithmic phase of growth when inositol is absent from the growth medium, *INO1* and other coordinately regulated genes are derepressed. If inositol is added to the growth medium, these genes are repressed and addition of choline when inositol is present leads to further repression. However, addition of choline by itself has little or no effect.

A repeated element (consensus 5′-CATGTGAAAT-3′) first detected in the promoter of the *INO1* gene (8,9) known as the inositol-sensitive upstream activated sequence (UAS_{INO}) has been detected in the promoters of all such co-regulated genes of *S.cerevisiae* (1–3). This element is the binding site for a heterodimer composed of the products of the *INO2* and *INO4* regulatory genes (9–12). The *INO2* and *INO4* gene products (Ino2p and Ino4p) contain the basic helix–loop–helix (bHLH) DNA binding motif and their binding site, UAS_{INO}, contains within it the canonical bHLH site: CANNTG. Strains containing *ino2* and *ino4* mutations are inositol auxotrophs and display other abnormalities of phospholipid metabolism due to failure to derepress the *INO1* gene and other co-regulated genes of lipid metabolism (13). Mutations at a third locus, *OPI1*, lead to constitutive overexpression of *INO1* and a consequent overproduction of inositol (Opi^-) phenotype (14). The *OPI1* gene product also exerts its effect through UAS_{INO} (11) but it has not yet been established whether the effect of the *OPI1* gene product (Opi1p) on the co-regulated genes is direct or indirect (15). Opi1p contains within it a leucine zipper and polyglutamine stretches (16), both of which are motifs that are commonly found in DNA binding proteins, but Opi1p does not appear to bind directly to DNA (15).

Phospholipid biosynthesis in general decreases and phospholipid biosynthetic enzymes are repressed when yeast cells enter stationary phase (17). Yeast cells exhibit many responses as they enter stationary phase, including arrest in an unbudded state, changes in cell wall structure and accumulation of storage carbohydrates (18). Metabolic changes resembling those observed upon entry into stationary phase are observed when yeast cells growing in the presence of glucose are starved for an essential nutrient such as nitrogen, phosphorus or sulfur (19). These changes have been proposed to be responses to a signal transduction pathway called the fermentable growth medium (FGM)-induced pathway (20). Since the *INO1* gene is repressed as cells approach stationary phase in glucose-containing medium (21–23), even when inositol is absent, we explored the response of *INO1* to transient and total nitrogen limitation in the presence of glucose and other essential nutrients. We report that *INO1* promoter is exquisitely sensitive to nitrogen limitation and that this response is mediated by UAS_{INO} and requires the participation of Opi1p. Furthermore, repression of the *INO1* gene in response to nitrogen limitation, similar to its repression in response to the presence of inositol, requires ongoing phosphatidylcholine synthesis.

MATERIALS AND METHODS

Materials

Sources of materials were: [α-^{32}P]cytidine 5′-triphosphate (sp. act. 800 Ci/mmol), DuPont NEN; nitrocellulose, Schleicher & Schull; SP6/T7 Transcription Kit, Boehringer Mannheim. All other materials were reagent grade or better.

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Strains and plasmids used

The genotypes of the strains of *S.cerevisiae* yeasts used in this study are described in Table 1. Construction and description of plasmid vectors for heterologous reporter gene expression driven by fragments of the *INO1* promoter can be found in Lopes *et al*. (5,24) (pJH334 and pKS102) and Slekar and Henry (25) (pKH200). *INO1–lacZ* construct pJH334 contains ~1 kb of *INO1* sequence including 132 amino acids from the I-1-P synthase N-terminus and 543 nt 5′ to the start of transcription ligated to *lacZ*. This construct was linearized and integrated into the *ura3* locus of strain W303-1A to create BRS1069 (5). Construct pKS102 is an episomal *INO1–CYC1–lacI′Z* plasmid containing nt –259 to –154 of the *INO1* 5′ region (5). Construct pKH200 contains sequences identical to the first native 10 bp UAS_{INO} element (corresponding to the first UAS_{INO} on the pKS102 plasmid) placed upstream of the heterologous reporter gene *CYC–lacI′Z* (25). Yeast transformation was performed by the lithium acetate method (26) with minor modifications.

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source/reference
W303-1A	MAT**a** *leu2 his3 trp1 ura3 ade2 can1*	R. Rothstein
BRS 1021	MAT**a** *ade5 leu2 trp1 ura3 opi1*Δ	(16)
DC5	MAT**a** *leu2 his3*	J. Broach
WT-lacZ	MAT**a** *HIS3* [pVJ103, *INO1′*lacZ] *his3*	(21)
OP-lacZ	MAT**a** *opi1*Δ::*LEU2 leu2 HIS3* [pVJ103, *INO1′*lacZ] *his3*	(21)
BRS 1069	MAT**a** *ade2 his3 leu2 can1 trp1 ura3*::*INO1*-lacZ::*URA3* [pJH334]	(5)
Cho2 #50	MAT**a** *ade2 his3 ura3 leu2 cho2*::*LEU2*	(22)

Culture conditions

Yeast strains were routinely maintained on YEPD medium (1% yeast extract, 2% Bactopeptone, 3% glucose). Strains containing episomal plasmids were maintained and grown on vitamin defined yeast synthetic medium, YNBv, as described below, with the omission of uracil to maintain selective pressure. Synthetic medium with yeast nitrogen base and vitamins (YNBv) contained 30 g/l glucose, 5 g/l ammonium sulfate, 1 g/l potassium phosphate monobasic, 0.5 g/l magnesium sulfate, 0.1 g/l sodium chloride, 0.1 g/l calcium chloride, trace elements and vitamins as previously described (27) and the following mixture of amino acids and bases: 20 mg/l adenine, 20 mg/l arginine, 20 mg/l histidine, 60 mg/l leucine, 230 mg/l lysine, 20 mg/l methionine, 300 mg/l threonine, 20 mg/l tryptophan and 40 mg/l uracil. The above medium does not contain inositol. Where indicated, media were supplemented with 1 mM choline (C^+). Medium without ammonium sulfate (YNBv–NH_4) contained all components as above described for YNBv medium but ammonium sulfate was omitted. Medium with ammonium sulfate but without the mixture of amino acid and bases is referred to as YNBv–AA. Medium without ammonium sulfate and without the mixture of amino acids and bases listed above is designated YNBv–NH_4–AA.

All cultures were grown aerobically at 30°C with shaking. In a typical experiment, the culture was grown to early exponential phase of growth in YNBv medium, cells were collected by filtration, washed and resuspended in medium lacking one of the nutrients (ammonium sulfate, total nitrogen or phosphate). At indicated time points samples were collected and total RNA was isolated.

RNA analyses

RNA probes for northern blot hybridization were synthesized according to the manufacturer's recommendations for the SP6/T7 Transcription Kit (Boehringer Mannheim) from plasmids described in Hudak *et al*. (28), linearized with a restriction enzyme and transcribed with RNA polymerase as follows (plasmid/restriction enzyme/RNA polymerase): pAB309Δ/*Eco*RI/SP6 (*TCM1*); pJH310/*Hin*dIII/T7 (*INO1*); pTC101/*Eco*RI/SP6 (*lacZ*). RNA was isolated from yeast using glass bead disruption and hot phenol extraction (29). Northern hybridization was performed essentially as described by Hirsch and Henry (4) and the results were visualized by autoradiography and/or quantified using an AMBIS 4000 PhosphorImager (AMBIS Inc.) or densitometry. The quantity of *INO1* or *lacZ* RNA was normalized against *TCM1* RNA, as described previously (22).

RESULTS

Response of the *INO1* gene to nitrogen limitation

Since *INO1* gene expression is repressed in wild-type cells, even in the absence of inositol, as they enter stationary phase (21), we reasoned that starvation for essential nutrients might be responsible for this effect. To explore the response of the *INO1* gene to nitrogen limitation, wild-type strain W303-1A was grown in vitamin defined synthetic yeast medium without inositol containing 5 g/l ammonium sulfate (YNBv). Early in the exponential phase of growth, yeast cultures were transferred by filtration, as described in Materials and Methods, to growth medium lacking ammonium sulfate (YNBv–NH_4). Both YNBv and YNBv–NH_4 media contain the mixture of amino acids and bases, described in Materials and Methods. The response of the wild-type (W303-1A) yeast culture to the change from a readily used nitrogen source (ammonia) to poorly used nitrogen sources (supplied by the mixture of amino acids and bases) (30) was a slower growth rate (Fig. 1A). However, this shift produced only a minor change in the final density achieved by the culture after prolonged growth and there was no apparent cell cycle arrest during this shift as assessed by ratio of budded versus unbudded cells in the course of the shift (data not shown).

Expression of the *INO1* gene was strongly repressed immediately following the shift to the alternative nitrogen source (Fig. 1B). This response was transient, however, since *INO1* mRNA levels had begun to rise within 4 h and had returned to their original levels ~7 h after the shift. Presumably, this represents the period of time necessary for adjustment of the culture to the use of the alternative nitrogen sources. During the period of time following the shift to YNBv–NH_4 medium while *INO1* expression was repressed, there was relatively little effect on the transcript of the *TCM1* (ribosomal protein gene) used as a control for RNA loading (Fig. 1B). Since the product of the *OPI1* gene (Opi1p) is required for repression of *INO1* gene expression in response to inositol (11,16), we tested the level of *INO1* transcript in strain BRS1021, which contains an *opi1* deletion mutation (see Table 1 for complete strain descriptions). In this strain, unlike the wild-type, no repression of the *INO1* gene was observed in response to nitrogen limitation when the culture was transferred from YNBv to YNBv–NH_4 medium (Fig. 2).

INO1 expression was also studied during nitrogen starvation when no alternative nitrogen source was available. A wild-type strain requiring no amino acids (WT-lacZ) and a congenic strain

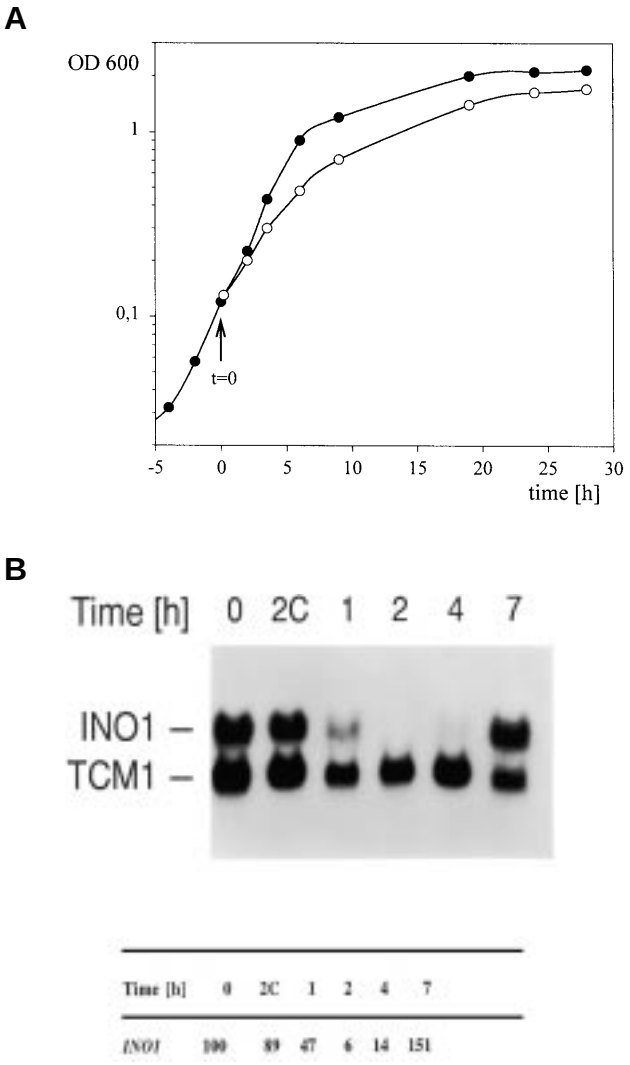


Figure 1. (**A**) Growth of the wild-type strain (W303-1A) after transfer to medium containing a poorly used nitrogen source. Cultures were grown in YNBv medium to logarithmic phase ($t = 0$). At $t = 0$, the culture was collected by filtration and washed with the medium to which it was to be transferred. One portion of the culture (○) was transferred to YNBv–NH_4 lacking ammonium sulfate. The other portion was transferred to fresh YNBv medium (●). Growth was monitored by optical density. (**B**) Northern blot analysis of *INO1* expression after transfer to medium containing a poorly used nitrogen source. At indicated time points after the transfer to YNBv–NH_4 medium, total RNA was isolated from collected samples and analyzed as described in Materials and Methods. Hybridization with the *TCM1* ribosomal protein gene probe serves as an RNA loading control. Time point 2C represents the control, at 2 h after transfer of one aliquot of the culture to fresh YNBv. Quantitation of *INO1* expression obtained by densitometry is shown below the blot. The proportion of *INO1* expression relative to the *TCM1* loading control was set at 100 at time 0. The numbers corresponding to subsequent times represent the percentage of *INO1* expression remaining relative to time 0.

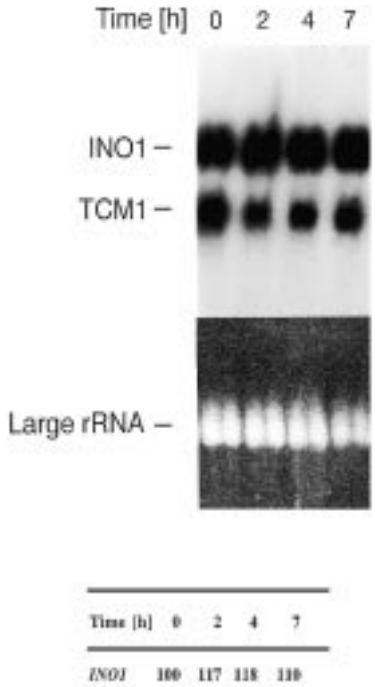


Figure 2. Analysis of *INO1* expression in an *opi1* mutant (BRS1021) after transfer to medium containing a poorly used nitrogen source. At indicated time points after shift from medium containing ammonium sulfate (YNBv) to medium containing only a poorly used nitrogen source (YNBv–NH_4), total RNA was isolated and subjected to northern blot analysis. Ethidium bromide staining of total RNA and hybridization with the *TCM1* probe serve as RNA loading controls. Quantitation of *INO1* expression obtained by densitometry is shown below the blot. The proportion of *INO1* expression relative to the *TCM1* loading control was set at 100 at time 0. The numbers corresponding to subsequent times represent the percentage of *INO1* expression remaining relative to time 0.

containing the *opi1* deletion mutation (OP-lacZ) (21) were grown to the early exponential phase of growth and transferred from synthetic medium (without inositol) containing ammonium sulfate (5 g/l) but no amino acids (YNBv–AA) to medium without ammonium sulfate and containing no other alternative source of nitrogen (YNBv–NH_4–AA). Both the wild-type strain and the *opi1* mutant underwent growth arrest very shortly after the shift to medium without any nitrogen source (data not shown). *INO1* gene expression was immediately and strongly repressed in the wild-type strain (Fig. 3) but remained derepressed in the congenic *opi1* derivative (Fig. 4). The *TCM1* gene was also repressed in both strains immediately after the transfer of culture to the medium without any nitrogen source. The repression of *TCM1* expression under conditions of total nitrogen withdrawal (Figs 3 and 4) is in sharp contrast to the continuing expression of *TCM1* during transient nitrogen starvation in both the *opi1* and wild-type strains (Figs 1 and 2). It is noteworthy, however, that *INO1* expression continued even after growth arrest in the *opi1* strain starved for nitrogen (Fig. 4). This is consistent with the report by Jiranek *et al.* (21) that *INO1* expression continues in *opi1* mutants well into stationary phase.

Ongoing phosphatidylcholine synthesis is essential for *INO1* repression due to nitrogen limitation

cho2 mutants, which are defective in the first methylation step en route to phosphatidylcholine (PC) (the pathway for phospholipid biosynthesis is shown in Fig. 5), and other mutants with defects in the methylation pathway leading to PC, display altered regulation of *INO1* in response to the soluble precursors of phospholipid biosynthesis, inositol and choline (22,31,32). These

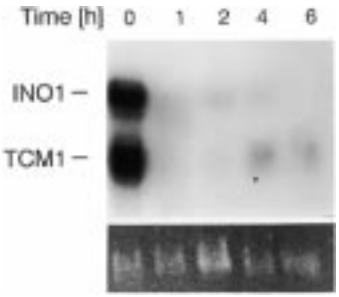


Figure 3. Northern blot analysis of *INO1* expression after transfer of culture to medium with no alternative nitrogen source. At indicated time points after transfer of the prototrophic wild-type strain, WT-lacZ, from defined synthetic yeast medium without amino acids (YNBv–AA) to medium without any nitrogen source (YNBv–NH_4–AA), total RNA was isolated and subjected to northern blot analysis. Ethidium bromide staining of total RNA was used as a control for RNA loading.

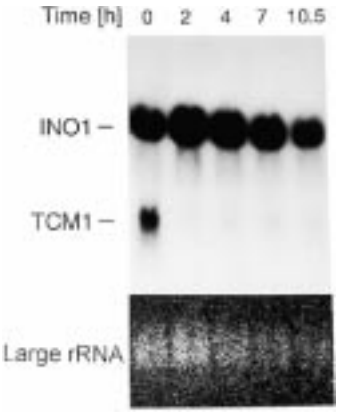


Figure 4. Northern blot analysis of *INO1* expression of an *opi1* strain after transfer of culture to medium without any nitrogen source. A prototrophic *opi1* strain (OP-lacZ) was transferred from vitamin defined synthetic yeast medium without amino acids (YNBv–AA) to medium without any nitrogen source (YNBv–NH_4–AA). At indicated time points after transfer total RNA was isolated and subjected to northern blot analysis. Ethidium bromide staining of total RNA serves as a loading control.

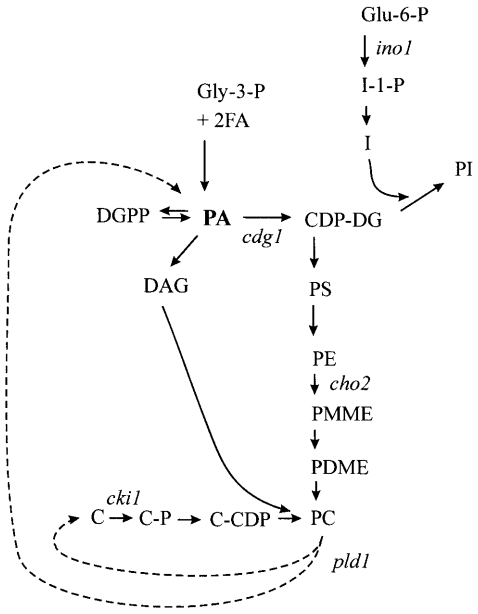


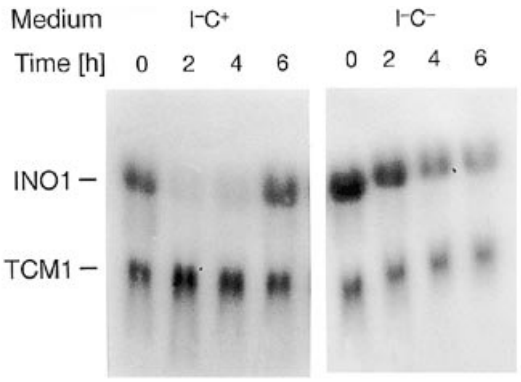
Figure 5. Phospholipid biosynthetic pathways in *S.cerevisiae*. The pathways shown include the relevant steps discussed in the text. Detailed descriptions are given elsewhere (2,3,7). Precursors and lipids: C, choline; C-CDP, cytidine diphosphate choline; CDP-DG, cytidine diphosphate diacylglycerol; C-P, choline phosphate; DAG, diacylglycerol; DGPP, diacylglycerol pyrophosphate; FA, fatty acid; Glu-3-P, glucose 3-phosphate; Gly-3-P, glycerol 3-phosphate; I, inositol; I-1-P, inositol 1-phosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMME, phosphatidylmonomethylethanolamine; PS, phosphatidylserine. The designations for the recessive mutant alleles (*italic*) rather than the wild-type structural genes are given: *cdg1*, CDP-DG synthase; *cho2* (also known as *pem1*), phospholipid N-methyltransferase; *cki1*, choline kinase; *ino1*, inositol 1-phosphate synthase pld1 (also known as *spo14*) phospholipase D. Dashed line, degradation of PC via phospholipase D-mediated route.

mutants have an inositol excretion phenotype (Opi^-) (14) that is eliminated when choline is supplied in the growth medium (22,32). The *cho2* mutants exhibit derepressed *INO1* levels even in the presence of inositol unless they are supplied with exogenous choline (22). Repression of the *INO1* gene in response to inositol in *cho2* mutants is restored by supplementation with the precursors monomethylethanolamine (MME) and dimethylethanolamine (DME) (32) which can be incorporated via the CDP choline pathway (33) into phospholipid. Since the *cho2* mutation blocks the conversion of phosphatidylethanolamine to phosphatidylmonomethylethanolamine, all three precursors enter phospholipid biosynthesis downstream of the *cho2* genetic lesion and restore PC biosynthesis (Fig. 5).

In the present study, the *cho2* strain was grown in defined yeast synthetic medium lacking inositol but containing ammonium sulfate (YNBv), with (C^+) or without (C^-) 1 mM choline supplement as indicated. Early in the exponential phase of growth, cultures were transferred to the respective media without ammonium sulfate but containing the mixture of amino acids and bases (YNBv–NH_4). In the presence of choline, *INO1* expression in the *cho2* mutant follows the same general pattern of transient repression of *INO1* expression in the absence of ammonium sulfate in the medium (YNBv–NH_4) (Fig. 6) that is observed in the wild-type strain (Fig. 1B). The repression is not quite as complete as is observed in the wild-type strain. However, in the *cho2* strain, in the absence of choline (Fig. 6), the *INO1* gene exhibits much less repression than is characteristic of the wild-type response to nitrogen limitation or is observed in the *cho2* strain itself when choline is present.

Effect of elements within the promoter of the *INO1* gene

Previously, a construct containing 543 nt of the *INO1* promoter upstream of the translational start site was shown to be sufficient to drive regulated expression in wild-type yeast cells of a fusion construct containing the *Escherichia coli lacZ* gene. This construct is regulated like the native *INO1* gene in response to the availability of the precursors inositol and choline (5). We asked whether this construct also showed repression comparable to the



Medium	I-C+				I-C-			
Time [h]	0	2	4	6	0	2	4	6
INO1	100	29	36	99	100	93	77	69

Figure 6. Northern blot analysis of *INO1* expression in a *cho2* strain, following transfer to medium with a poorly used nitrogen source, in the presence and absence of choline. The *cho2* strain was grown in YNBv to mid-logarithmic phase with and without choline (C^+ and C^-, respectively). The cultures were then transferred by filtration from complete synthetic medium (YNBv) to medium without ammonium sulfate (YNBv–NH_4). Choline supplementation (C^+) or lack thereof (C^-) was maintained at transfer. At indicated time points after the transfer, total RNA was isolated and subjected to northern blot analysis. Hybridization with the *TCM1* ribosomal protein gene probe serves as an RNA loading control. Quantitation of *INO1* expression obtained by densitometry is shown below the blot. The proportion of *INO1* expression relative to the *TCM1* loading control was set at 100 at time 0. The numbers corresponding to subsequent times represent the percentage of *INO1* expression remaining relative to time 0.

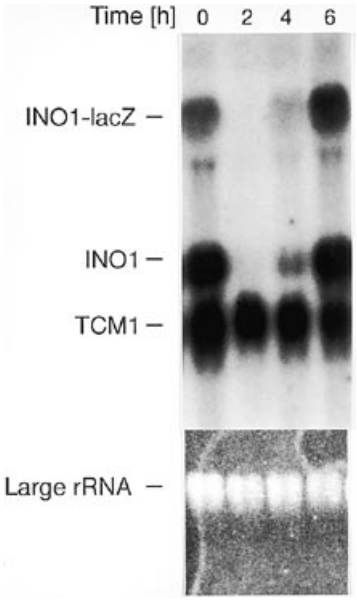


Figure 7. Expression of a construct (pJH334) containing the *lacZ* gene fused to the first 543 nt of the *INO1* promoter sequence, following transfer to medium containing a poorly used nitrogen source. Yeast strain BRS1069, containing construct pJH334 integrated at the *URA3* locus, was transferred by filtration from complete defined synthetic medium (YNBv) to medium without ammonium sulfate (YNBv–NH_4). At the indicated times following the transfer to new medium, total RNA was isolated and subjected to northern blot analysis. *INO1–lacZ* represents heterologous expression from an integrated plasmid driven by the first 543 nt of the *INO1* promoter. *INO1* represents expression of the native *INO1* gene. Hybridization with the *TCM1* probe and ethidium bromide staining of total RNA serve as loading controls.

native *INO1* transcript in response to transient nitrogen limitation upon shift to YNBv–NH_4 medium. Yeast strain BRS1069 contains an *INO1–lacZ* construct containing the first 543 nt of the *INO1* promoter 5′ to the *INO1* translation start site fused to *lacZ* and integrated at the *URA3* locus (5). This strain was transferred to YNBv–NH_4 medium following a protocol identical to that used in the experiments reported in Figure 1 for the wild-type strain (W303). Expression of the *lacZ* gene driven by the *INO1–lacZ* construct, as measured by northern blot, precisely mirrored expression of the native *INO1* transcript (Fig. 7).

To further characterize the *cis*-acting elements within the *INO1* promoter responsible for the regulation of the *INO1* gene in response to nitrogen limitation, we analyzed expression of fusion constructs containing smaller fragments of the *INO1* promoter. In these studies, *lacZ* expression was driven by fragments of the *INO1* promoter fused to *lacZ* contained on an episomal plasmid. Expression of *lacZ* measured by northern blot was compared to expression of the native *INO1* transcript in order to assess capability of specific regions within the *INO1* promoter to respond to the signal produced by nitrogen limitation upon shift from YNBv to YNBv–NH_4 medium. Both vectors (pKS102 and pKH200, as described in Materials and Methods) contain a portion of the *INO1* promoter fused to the *CYC–lacI′Z* chimera (5,25). Vector pKS102 contains promoter sequences –259 to –154 which include two active copies of the UAS_{INO} element from the *INO1* promoter (11,24,25), as well as a copy of an upstream repression sequence (URS1) (24), which has been reported in the promoters of many yeast genes (34). Construct pKH200 contains sequence identical to the 10 bp UAS_{INO} element found in position –244 to –235 of the *INO1* promoter (the first of two UAS_{INO} elements present in plasmid pKS102) (25). The pattern of transcription of the *lacZ* fusion construct driven by the fragments of the *INO1* promoter containing the two UAS_{INO} elements together with URS1 (pKS102) was similar to the response of the native *INO1* gene under the same conditions (Fig. 8). Similarly, *lacZ* transcription driven only by the first of these UAS_{INO} elements (pKH200) also mirrors the pattern of *INO1* expression of the native *INO1* transcript (Fig. 9). However, the absolute levels of *lacZ* expression were approximately three times higher from the pKH200 construct under both repressed and derepressed conditions as compared to *lacZ* mRNA levels derived from pKS102. The effect of nitrogen limitation on expression of these *INO1* constructs is similar to the effects obtained when these same constructs were studied in conjunction with repression in response to inositol (24).

DISCUSSION

Nutrient starvation is a fundamental condition that signals microorganisms to slow cellular metabolism, cease cell division and enter stationary phase. In glucose grown cells, starvation for nitrogen triggers the fermentable growth medium (FGM) signal transduction pathway which results in the appearance of stationary

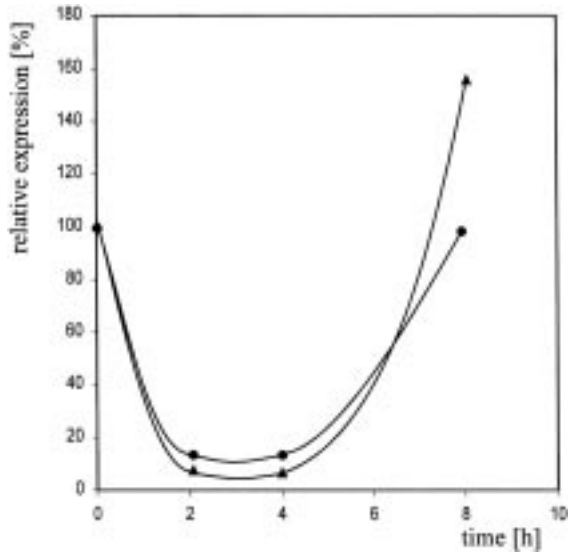


Figure 8. Expression driven by promoter fusions containing *cis*-acting elements from the *INO1* promoter, following transfer to medium with poorly used nitrogen sources. Yeast strain W303-1A was transformed with plasmid pKS102, grown to logarithmic phase in complete synthetic medium (YNBv) and transferred by filtration to medium without ammonium sulfate (YNBv–NH_4). At the indicated time points following transfer, total RNA was isolated and subjected to northern blot analysis. Northern blots were quantified as described in Materials and Methods. Expression of *lacZ* (●) and native *INO1* (▲) were normalized to *TCM1* as a loading control.

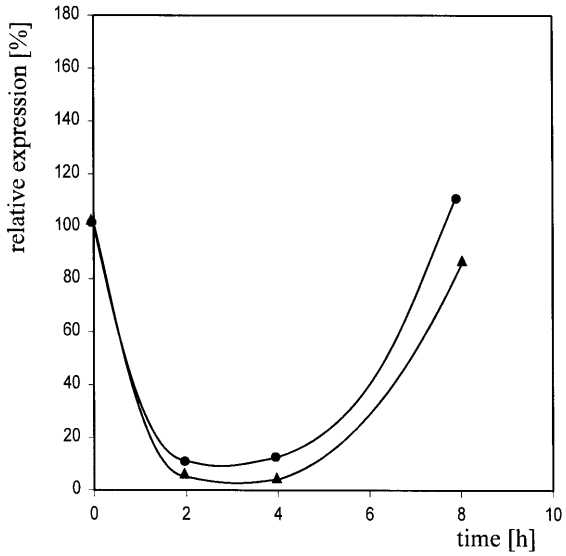


Figure 9. Expression of the *lacZ* gene driven by a UAS_{INO} element, following transfer to medium with poorly used nitrogen sources. Yeast strain W303-1A was transformed with plasmid pKH200 and transferred from complete synthetic medium (YNBv) to medium lacking ammonium sulfate (YNBv–NH_4). At the indicated time points following the transfer to new medium, total RNA was isolated and subjected to northern blot analysis. The RNA was quantified as described in Materials and Methods. *lacZ* (●) RNA and native *INO1* RNA (▲) were normalized to *TCM1* as a loading control.

phase characteristics. Nutrient-starved cells arrest in the G_1 phase of the cell cycle, enter G_0 and exhibit characteristics of stationary phase arrest including: glycogen and trehalose accumulation, repression of ribosomal protein genes and induction of heat shock genes. Re-feeding with the limiting nutrient results in rapid disappearance of the stationary characteristics (19). The coordination of cell growth and metabolism with membrane biogenesis requires that production of membrane constituents, including phospholipids, must be regulated at some level by the availability of basic nutrients such as nitrogen and phosphate. The *INO1* gene, the most highly regulated of the set of co-regulated genes of phospholipid biosynthesis (1–3) is derepressed during the active exponential growth in yeast cultures grown in medium lacking inositol. Homann *et al*. (17) demonstrated that the enzymes of the yeast phospholipid biosynthetic pathway are regulated in response to growth phase and Lamping *et al*. (23) and Jiranek *et al*. (21) demonstrated that this growth phase regulation occurs, at least partly, at the level of transcript abundance of the co-regulated genes, including *INO1*. As mitotically growing cultures approach stationary phase, the *INO1* gene is repressed even when inositol is absent (21,23). However, the *INO1* gene is derepressed during meiosis (34).

In the present study, we have followed expression of the *INO1* gene when yeast cells were challenged by limitation of nitrogen. Total absence of a nitrogen source caused rapid cessation of *INO1* transcription and also affected the levels of the *TCM1* ribosomal protein gene (Fig. 3). There is an expected response by the *TCM1* gene since repression of ribosomal protein genes in response to nutrient limitation has been well documented and is believed to be under the control of the FGM signal transduction pathway (18,19). However, when nitrogen was only temporarily unavailable during the adjustment of cellular metabolism to the use of alternative nitrogen sources (30), *INO1* transcription was repressed transiently while the level of the *TCM1* transcript was only slightly affected (Fig. 1). Thus, the response of the *INO1* gene to transient nitrogen limitation was more sensitive than the response of the *TCM1* gene. Furthermore, when the *opi1* gene is deleted, *INO1* expression continues even when nitrogen is completely absent from the medium (Fig. 4), but the *opi1* mutation has no effect on *TCM1* expression (compare Figs 3 and 4) under these conditions. These observations suggest that *INO1* repression in response to nutrient limitation is separate from the regulatory mechanism controlling the *TCM1* gene under these same conditions.

By studying *lacZ* fusion constructs whose expression was driven by portions of the *INO1* promoter, we have shown that the elements of the *INO1* promoter necessary for repression in response to inositol (UAS_{INO}) are also sufficient to drive repression of *INO1* in response to transient nitrogen limitation (Figs 7–9). Thus, it appears that *INO1* sensitivity to nitrogen limitation is controlled by UAS_{INO}, the same element that controls repression in response to inositol (5,9,35). The *INO1* promoter contains two active UAS_{INO} elements (5,9,35) and an active URS1 element (24). The *INO1* promoter has been extensively studied with respect to each of the UAS_{INO} elements and the URS1 element. In a previous study, binding of a putative URS binding factor with the URS1 element in the *INO1* promoter was analyzed and the activity was shown to be competed by the *CAR1* URS1 sequence (24). The URS1 element has been shown to affect the overall level of repression of *INO1*, but UAS_{INO} is solely responsible for mediating the response to inositol (11,24).

The presence of a single synthetic UAS_{INO} element in a completely heterologous reporter gene construct is completely sufficient to confer repression in response to inositol and to place

the construct under *OPI1* control (11). In previous studies, we have shown that the *OPI1* gene product is required not only for repression of *INO1* in response to inositol (11) but also for its repression as cells enter stationary phase (21). Expression of *INO1* is also very sensitive to mutations affecting transcription globally (reviewed in 2). For example, the *SIN3* gene product, which is a component of a large complex involved in histone deacetylation, also affects *INO1* expression (28). The effect of *sin3* mutations on *INO1* expression was shown to be mediated both by URS1 and UAS_{INO} (25). Most recently, *INO1* expression has also been shown to be influenced by two major signal transduction pathways, the unfolded protein response pathway (36) and the glucose response pathway (37,38). In both cases, mutations in protein kinases, *IRE1* and *SNF1/SNF4*, respectively, lead to loss of *INO1* expression and resulted in an Ino^- phenotype. In both cases, deletion of the *OPI1* gene restored *INO1* expression but not its regulation in response to inositol (36,37). Thus, the regulation of *INO1* is very complex and is influenced by the overall status of the cellular transcription apparatus and several major signal transducing pathways. In this report, we have shown that the *INO1* gene is rapidly repressed in response to nitrogen limitation and that this response appears to be controlled by the same mechanism that controls repression in response to inositol, namely the response is conferred by UAS_{INO} and requires a functioning *OPI1* gene product. The fact that deprivation of nitrogen triggers repression of *INO1* via the control of UAS_{INO} provides information relevant to potential mechanisms for the observed repression of UAS_{INO}-containing genes upon entry into stationary phase (21,23).

Griac *et al*. (22) showed that the kinetics of repression of UAS_{INO}-containing genes upon entry into stationary phase was altered in cells containing mutations affecting PC biosynthesis. Specifically, it was shown that *cho2* mutants, which have a defect in phospholipid methylation (Fig. 5), exhibited slower kinetics of repression upon entry into stationary phase when starved for choline (22). Yeast strains carrying the *cho2* mutation also show aberrant transcriptional regulation in response to inositol unless a metabolite downstream of the genetic block in PC biosynthesis is supplied exogenously (22,32). Specifically, *cho2* cells fail to repress the *INO1* gene and other co-regulated genes of phospholipid biosynthesis in response to inositol, unless choline (or MME or DME) is also supplied. In this study, we observed that when *cho2* cells were grown in the absence of choline, as well as inositol, and were subsequently transferred to medium lacking ammonium sulfate, as well as choline and inositol, the *INO1* gene did not exhibit transient repression in response to nitrogen limitation. However, when the same experiment was repeated with *cho2* cells grown in medium containing choline but lacking inositol, the *INO1* gene was repressed, following transfer to nitrogen limiting medium. Thus, growth of *cho2* cells in the absence of choline, a condition that results in reduced PC biosynthesis and an inability to repress the *INO1* gene in response to inositol, also results in inability of the cells to repress the *INO1* gene in response to nitrogen limitation. Restoration of PC biosynthesis by supplying exogenous choline restores repression in response to both signals, i.e. inositol (22) and nitrogen limitation (Fig. 6). This observation suggests that the metabolic signals for the two types of response (i.e. by *INO1* repression in response to inositol or to nitrogen limitation) may be identical. This connection between inositol and nitrogen metabolism is currently being explored.

We have recently proposed a model for the transcriptional regulation of the phospholipid biosynthetic genes which can account for repression in response to inositol/choline availability in logarithmically growing cells (2,40). Our model proposes that the build-up of a precursor early in phospholipid biosynthesis, either phosphatidic acid (PA) or a precursor closely related to PA (Fig. 5), produces a signal that results in derepression of the co-regulated genes containing UAS_{INO}. Cellular levels of PA are influenced by at least three ongoing metabolic processes: (i) the rate of *de novo* synthesis of PA from glycerol 3-phosphate and fatty acids (Fig. 5); (ii) the rate of production of PA by phospholipase D-mediated phospholipid turnover (40,41); (iii) the rate of utilization of PA in downstream reactions of phospholipid biosynthesis (42). Inositol, when available in the growth medium, draws on the pool of cytidine diphosphate diacylglycerol (CDP-DG) which, in turn, draws upon PA. Choline draws upon the availability of diacylglycerol which is, in turn, derived from PA (Fig. 5). Our model proposes that when the rate of PA production, via *de novo* synthesis and/or turnover of existing lipids, exceeds its utilization in downstream reactions, *INO1* and co-regulated UAS_{INO}-containing genes will be derepressed (2). The *cho2* mutation blocks the major pathway leading from PA through CDP-DG and phosphatidylserine to PC (Fig. 5). Consistent with the model, in *cho2* cells growing in the absence of choline, neither inositol (22) nor transient nitrogen limitation (Fig. 6) causes repression of *INO1*. However, when choline is supplied to *cho2* mutants, it permits the synthesis of PC drawing on diacylglycerol downstream of PA (Fig. 5) and simultaneously restores repression of *INO1*, both in response to inositol (22) and nitrogen deprivation (Fig. 6). The results depicted in Figure 6 clearly show that the metabolic signal that triggers *INO1* repression in response to transient nitrogen limitation, like the response to inositol availability, is influenced by the pattern of phospholipid metabolism.

The experiments reported here show that *INO1* is rapidly repressed in response to nitrogen limitation. Moreover, this regulation shares common features with the regulation that occurs in response to inositol and choline (8). (i) Repression in response to both types of metabolic signals (i.e. inositol and nitrogen limitation) are dependent on a functional *OPI1* gene product; (ii) ongoing PC biosynthesis is necessary for proper wild-type regulation in both cases; (iii) in both cases, the minimal promoter requirement for proper regulation of a heterologous system is the 10 bp UAS_{INO} consensus sequence. These common features suggest that a single regulatory mechanism is involved in controlling repression of the *INO1* gene to nitrogen limitation and inositol/choline availability. This hypothesis and the interconnection between inositol and nitrogen metabolism are currently being explored in our laboratory.

ACKNOWLEDGEMENT

Supported by grant GM 19629 to S.A.H. from the National Institutes of Health.

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