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Expression of *GUT1*, which encodes glycerol kinase in *Saccharomyces cerevisiae*, is controlled by the positive regulators Adr1p, Ino2p and Ino4p and the negative regulator Opi1p in a carbon source-dependent fashion

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ABSTRACT

In *Saccharomyces cerevisiae* glycerol utilization is mediated by two enzymes, glycerol kinase (Gut1p) and mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p). The carbon source regulation of *GUT1* was studied using promoter–reporter gene fusions. The promoter activity was lowest during growth on glucose and highest on the non-fermentable carbon sources, glycerol, ethanol, lactate, acetate and oleic acid. Mutational analysis of the *GUT1* promoter region showed that two upstream activation sequences, UAS_{INO} and UAS_{ADR1}, are responsible for ~90% of the expression during growth on glycerol. UAS_{ADR1} is a presumed binding site for the zinc finger transcription factor Adr1p and UAS_{INO} is a presumed binding site for the basic helix–loop–helix transcription factors Ino2p and Ino4p. *In vitro* experiments showed Adr1 and Ino2/Ino4 protein-dependent binding to UAS_{ADR1} and UAS_{INO}. The negative regulator Opi1p mediates repression of the *GUT1* promoter, whereas the effects of the glucose repressors Mig1p and Mig2p are minor. Together, the experiments show that *GUT1* is carbon source regulated by different activation and repression systems.

INTRODUCTION

Like many microorganisms, *Saccharomyces cerevisiae* has developed complex systems which allow it to respond quickly to changes in nutrient composition. In general, genes necessary for utilization of certain nutrients are repressed when a more favorable substrate is available. An example of this is glucose repression (carbon catabolite repression) where the presence of the rapidly fermentable sugar glucose represses a large number of genes. Glucose-repressed genes include those that are essential for catabolism of slowly fermentable sugars, gluconeogenesis, the tricarboxylic acid cycle and respiration (for a recent review see 1). We have focused our attention on the effect of different carbon sources on expression of the glycerol kinase gene, *GUT1*. This study was prompted by the identification of regulatory elements in the *GUT1* promoter previously shown to mediate transcriptional regulation of other yeast genes.

Saccharomyces cerevisiae is capable of using glycerol as the sole source of carbon and energy. Glycerol can permeate the plasma membrane by passive diffusion (2), facilitated diffusion through the Fps1p channel protein (3), and by a glycerol/proton symport system (4). Glycerol degradation occurs via a phosphorylative pathway (5). In the first step, glycerol is converted to glycerol-3-phosphate by cytosolic glycerol kinase (EC 2.7.1.30). Glycerol-3-phosphate then passes the outer mitochondrial membrane and is oxidized to dihydroxyacetone phosphate (DHAP) by an inner mitochondrial membrane enzyme, FAD-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.99.5). DHAP returns to the cytosol, where it is either catabolized in glycolysis or used for synthesis of glucose-6-phosphate during gluconeogenesis. The structural genes encoding glycerol kinase (*GUT1*) and FAD-dependent glycerol-3-phosphate dehydrogenase (*GUT2*) have been cloned, and show similarity to prokaryotic and eukaryotic homologs (6–8). The inability of *gut1* and *gut2* mutants to use glycerol as sole source of carbon and energy (5) suggests that the phosphorylative pathway is the major route for glycerol assimilation in *S.cerevisiae*.

During growth on non-fermentable carbon sources, the cell requires functional *ADR1* for expression of *GUT1*, *ADH2* (catabolic alcohol dehydrogenase), *ACS1* (acetyl-CoA synthetase) and genes involved in peroxisome proliferation such as *CTA1*, *FOX2*, *FOX3* and *PAS2* (6,9–11). Glucose diminishes Adr1p by decreasing the translation of *ADR1* mRNA (12). Adr1p is a C_2H_2 zinc finger protein, which normally binds as two monomers to two inverted repeats non-cooperatively (13,14). However, Adr1p-mediated transcriptional activation through a single Adr1p binding site has been reported for expression of the *Hansenula polymorpha* methanol oxidase gene (*MOX*) in *S.cerevisiae* (15). Deletion studies of Adr1p have identified several functional regions, including a region necessary for growth on glycerol (16).

Gut1p phosphorylates glycerol to glycerol-3-phosphate, which is an early intermediate in synthesis of phospholipids (17,18). Many genes involved in biosynthesis of fatty acids and phospholipids are coordinately regulated by the *trans*-acting

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Table 1. Yeast strains used in this study

Strain	Genotype	Source or reference
W303-1A	*MAT***a** *ade2-1 his3-11 leu2-3,112 can1-100 trp1-1 ura3-1*	(55)
BRS2001[a]	*MAT***a** *ino2*Δ*::TRP1 ade2-1 his3-11 leu2-3,112 can1-100 trp1-1 ura3-1*	Lopes laboratory
BRS2004[a]	*MAT*α *ino4*Δ*::LEU2 ade2-1 his3-11 leu2-3,112 can1-100 trp1-1 ura3-1*	Lopes laboratory
BRS2005[a]	*MAT***a** *opi1*Δ*::LEU2 ade2-1 his3-11 leu2-3,112 can1-100 trp1-1 ura3-1*	(56)
W303-1A(*INO2-cat*)[a]	*MAT***a** *gal4::INO2*p-*cat ade2-1 his3-11 leu2-3,112 can1-100 trp1-1 ura3-1*	(26)
CEN.PK113-5D	*MAT***a** *ura3-52 MAL2-8*^{c} *SUC2*	Scientific Research & Development GmbH, Oberusel, Germany
T453[b]	*MAT***a** *mig1::MEL1 ura3-52 MAL2-8*^{c} *SUC2*	(57)
T470[b]	*MAT***a** mig1::*MEL1 mig2::URA3 ura3-52 MAL2-8*^{c} *SUC2*	Danisco Biotechnology
B290[b]	*MAT***a** *mig1::MEL1 mig2::URA3 MAL2-8*^{c} *SUC2 ura3*	This study

[a]Parental strain W303-1A.
[b]Parental strain CEN.PK113-5D.

regulatory proteins Ino2p, Ino4p and Opi1p (reviewed in 18). The *GUT1* promoter also includes a potential Ino2p/Ino4p binding site. Ino2p and Ino4p are members of the basic helix–loop–helix (bHLH) family of DNA binding proteins. *In vitro* studies have shown that Ino2p and Ino4p heterodimerize and bind a *cis*-acting promoter element (5′-CATGTGAAAT-3′) called UAS_{INO} (upstream activation sequence) (19–21). UAS_{INO} has been shown to function in both orientations (22), and is the same element as the ICRE element found in the fatty acid synthase genes *FAS1* and *FAS2* (23). Mutational analyses have shown that the core bHLH binding site (CATGTG) found in UAS_{INO} is required for its function as a UAS (21,23). The Ino2p/Ino4p:UAS_{INO} system is required for derepression of a large number of genes in response to inositol and choline deprivation in addition to, for example, nitrogen availability (18,24). Opi1p negatively controls expression of Ino2p/Ino4p-regulated genes by an as yet unknown mechanism (25,26). Recently, it was shown that the Snf1p-Snf4p protein kinase and the Glc7p-Reg1p protein phosphatase, which are components of the general glucose repression pathway, are also involved in repression of Ino2p/Ino4p target genes (27,28).

This study reports the analysis of *GUT1* expression under different growth conditions. Expression of the gene was found to be derepressed when cells were grown on non-fermentable carbon sources (e.g. glycerol and ethanol) and repressed on fermentable carbon sources such as glucose. Two *cis*-acting elements, UAS_{ADR1} and UAS_{INO}, were required for >90% of the expression during growth on glycerol. Mobility shift assays showed that both Adr1p and Ino2p/Ino4p bound to the cognate *cis*-acting elements in the *GUT1* promoter. Glucose repression was partly relieved in an *opi1* mutant (~20-fold). This observation identifies a link between carbon and phospholipid metabolism. In addition, glucose repression was slightly relieved in *mig1* and *mig2* mutants (~2-fold).

MATERIALS AND METHODS

Strains and media

The *S.cerevisiae* strains used in this study are listed in Table 1. *Escherichia coli* strain DH5α (29) was used for transformation and plasmid amplification, and M15pREP4 (30) was used for expression of the Adr1 polypeptide used for mobility shift assay.

Yeast was cultured in yeast extract–dextrose (YPD) or synthetic complete (SC) medium (31) lacking uracil. SC was supplied with either 0.2 or 2% (w/v) glucose, 2% (w/v) galactose, 2% (w/v) raffinose, 3% (v/v) glycerol, 3% (v/v) ethanol, 3% (v/v) DL-lactate, 0.2% (v/v) oleic acid or 3% (w/v) acetate. Bacteria were cultured in 2× YT medium or LB (32) supplied with 25 mg/l kanamycin and/or 100 mg/l ampicillin. I^+C^+ and I^-C^- media (33), used to investigate the influence of inositol and choline on *GUT1–lacZ* expression, were supplemented with the same concentrations of amino acids and adenine as used in SC medium and either 2% (w/v) glucose or 3% (v/v) glycerol.

Isolation of a *mig1 mig2* double mutant

A *mig1 mig2* mutant (B290) without orotidine-5′-phosphate decarboxylase activity (*ura3*) was isolated by plating T470 (*mig1 mig2*) on 5-FOA medium (34).

Recombinant DNA methods, plasmid construction and transformation

Standard recombinant DNA methods were carried out as described by Sambrook *et al.* (32).

For construction of a *GUT1–lacZ* translational fusion plasmid (pMG29), 1002 bp of the *GUT1* promoter were amplified by PCR, using the oligonucleotides 5′-GCGCGGATCCA-GACAAGCAACC-3′ and 5′-GCGCGGATCCATATAACT-ATTTGTATAGTT-3′ containing *Bam*HI restriction sites (underlined) which allow in-frame fusion of the first codon in *GUT1* with the seventh codon in *lacZ*. The *Hin*dIII + *Bam*HI-digested PCR fragment of 744 bp was inserted in-frame with *lacZ* in the *ARS1–CEN4–URA3* plasmid pFN8 (35).

GUT1–lacZ plasmids with mutations in the *cis*-acting elements UAS_{ADR1}, UAS_{INO} and URS_{MIG} were constructed (Fig. 3) by inserting a 744 bp *Hin*dIII–*Bam*HI *GUT1* promoter fragment from pMG29 into pUC19 (36) and mutating with the QuikChange™ Site-Directed Mutagenesis system (Stratagene). Mutations were confirmed by DNA sequencing and digestion with restriction enzymes and mutated promoter fragments were then ligated as *Hin*dIII–*Bam*HI fragments into pFN8. The

following oligonucleotides with mutations (bold) and restriction sites (underlined) were used for mutagenesis of putative *cis*-acting elements in the *GUT1* promoter together with the oligonucleotide binding the opposite strand. The position relative to the start codon in the *GUT1* promoter and names of the resulting plasmids are indicated in parentheses. 5′-GGTGTAATAAAATG**ATATC**GGATGCCTGTTCTCG-3′ (–91 to –57 bp, pMG37), 5′-CTGTTTTTGTTTTT**GGGCCC**GTAAATAACGAC-3′ (–332 to –297 bp, pMG41, pMG49 and pMG77), 5′-CTCTTCTAATT**TTAAA**AAAACCATCAATTAAAGGG-3′ (–230 to –196 bp, pMG38, pMG49, pMG65 and pMG77), 5′-CAATTAAAGGGTG**CATGC**TAGCATAGTG-3′ (–206 to –179 bp, pMG64, pMG65, pMG76 and pMG77), 5′-CGACCGTCT**GTACTT**TAAAGCCTGGG-3′ (–521 to –496 bp, pMG75 and pMG77), 5′-CATCTCCCAAGC**CTCGAGCC**CTCGGTCGCAG-3′ (–512 to –482 bp, pMG75, pMG76 and pMG77).

Yeast was transformed by electroporation (37).

Enzyme assays

To measure β-galactosidase activity, transformants were inoculated from overnight (fermentable carbon sources) or 48 h (non-fermentable carbon sources) cultures and grown to an OD_{600} between 0.8 and 1.2, after which the cells were harvested, made permeable and assayed as described by Didion *et al.* (38). β-Galactosidase activity is indicated in Miller units [1000 × absorbance at 420 nm, divided by OD_{600} for the culture, volume of permeabilized cells (ml) and time (min)]. Chloramphenicol acetyltransferase (CAT) activity was measured by determination of the amount of acetylated chloramphenicol by the phase extraction method (39) as described by Ashburner and Lopes (26). CAT activity is indicated as per cent of total counts per minute in the organic phase divided by the amount of protein (μg) and time (h).

All enzymatic values are from at least three measurements of independent transformants.

RNA analysis

Total RNA from cultures with an OD_{600} of 0.8–1.2 was isolated according to the method of Schmitt *et al.* (40). Twenty micrograms of RNA were separated on a 1% agarose–2.2 M formaldehyde gel, bound to Hybond N membranes and hybridized overnight at 65°C according to the supplier (Amersham). *GUT1* mRNA was probed with a 2.0 kb *Spe*I–*Xba*I fragment and *ACT1* mRNA with a 3.8 kb *Eco*RI–*Bam*HI fragment, uniformly labeled with [α-^{32}P]dCTP, using the Ready To Go labeling kit (Pharmacia). Quantification was performed on an InstantImager (ΔPackard).

Electrophoretic mobility shift assays (EMSA)

Whole yeast cell extract was prepared from W303-1A (*INO2 INO4*) cells grown in I^-C^- medium and BRS2001 (*ino2*) and BRS2004 (*ino4*) cells grown in I^+C^+ medium. Both media contained 2% glucose. Total protein was extracted as described by Lopes and Henry (20), except that the protein was precipitated with $(NH_4)_2SO_4$ to 60% final concentration. Expression of the DNA binding domain of Adr1p (amino acids 17–162) was done in *E.coli* strain M15[pREP4] transformed with plasmid pQE31 (kindly provided by Dr Dombek, University of Washington). *Escherichia coli* cell extract was made from a 2× YT culture, induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) at an OD_{600} of 0.6 and harvested 5 h after induction.

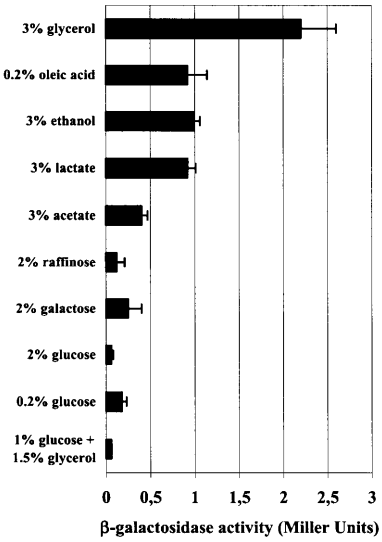

Figure 1. *GUT1–lacZ* expression on different carbon sources in W303-1A cells transformed with the *GUT1–lacZ* reporter plasmid (pMG29) and grown in media with the indicated carbon sources. Specific β-galactosidase activities are given in Miller units. Error bars indicate standard deviations of mean values.

Cell extract was dissolved in protein buffer (20 mM HEPES pH 8.0, 5 mM EDTA, 20% glycerol). Oligonucleotides harboring UAS_{INO} and UAS_{ADR1} (–221 to –189 bp) from the *GUT1* promoter were annealed as described by Lopes and Henry (20). 5′-CTGTTTTTGTTTTTCACATGGTAAATAACGA-3′, harboring UAS_{INO}, was annealed to 5′-TCGTTATTTACCATGTGAAAAACAAAAACAG-3′, and 5′-AATTGGAGTAAAACCATCAATTAAAGGGTGTGGAGTAGC-3′, harboring UAS_{ADR1}, was annealed to 5′-GCTACTCCACACCCTTTAATTGATGGTTTTACTCCAATT-3′. Annealed oligonucleotides were radiolabeled at the 5′-end with [γ-^{32}P]ATP as described by Sambrook *et al.* (32). Standard binding reactions were carried out in a total volume of 20 μl containing 4 mM Tris–HCl pH 8.0, 4 mM $MgCl_2$, 4% glycerol, 10 mM dithiothreitol, 25 mM KCl, 1 μg poly(dI·dC) (Amersham), 5 nM radiolabeled double-stranded oligonucleotide and 2 or 10 μg of either *E.coli* or yeast cell extract. Protein–nucleic acid complexes were allowed to form at 30°C for 10 min before separation (without dye solution) on a 4% acrylamide gel.

RESULTS

GUT1–lacZ expression on different carbon sources

Sprague and Cronan (5) have shown that glycerol kinase activity in *S.cerevisiae* is regulated according to carbon source. To investigate if this regulation occurred at the transcriptional level, a *GUT1–lacZ* translational reporter plasmid was constructed (pMG29). *GUT1–lacZ* expression was measured by determining β-galactosidase activity in transformed cells grown in media with fermentable and non-fermentable carbon sources (Fig. 1). The highest level of β-galactosidase activity was observed in glycerol-grown cultures. After growth in media with other non-fermentable carbon sources (ethanol, lactate and oleic acid) the level of β-galactosidase activity decreased by 50–60% compared with glycerol-grown cells

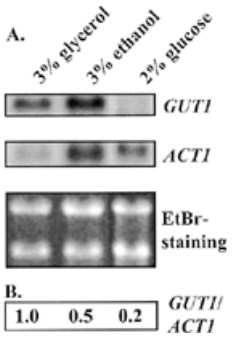

Figure 2. (**A**) Northern analysis of *GUT1* mRNA levels during growth on different carbon sources. Twenty micrograms of total RNA from W303-1A cells were separated, blotted and hybridized to *GUT1*-specific and *ACT1*-specific ^{32}P-labeled probes. (Below) Ethidium bromide stained gel. (**B**) Quantification of the northern blot depicted in (A), indicated as the ratio between *GUT1* and *ACT1* transcripts.

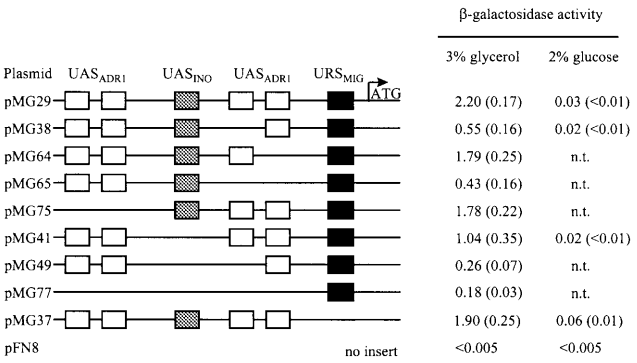

Figure 3. Effect of mutations in *cis*-acting elements identified in the *GUT1* promoter. W303-1A cells were transformed with a *GUT1–lacZ* reporter plasmid with 744 bp of either wild-type *GUT1* promoter (pMG29) or *GUT1* promoter with mutations in different *cis*-acting elements. UAS$_{INO}$ (shaded boxes), URS$_{MIG}$ (filled boxes) and/or left and right GGAG elements in the two UAS$_{ADR1}$ (open boxes). Mutations introduced in the *cis*-acting elements are indicated by the lack of a box. Specific β-galactosidase activities are given in Miller units. Standard deviations of mean values are given in parentheses.

(Fig. 1). Growth on acetate or galactose resulted in ~10–20% of the β-galactosidase activity observed in glycerol cultures. Very low β-galactosidase activity could be observed in glucose-grown cells, at both high (2%) and low (0.2%) glucose concentrations. A comparison of *GUT1–lacZ* expression on glycerol and on glucose showed that *GUT1–lacZ* expression in glycerol-grown cultures was ~75-fold higher compared with glucose-grown cultures. To test if glycerol is able to induce *GUT1–lacZ* expression in the presence of glucose, the cells were inoculated in medium containing a mixture of 1.5% glycerol and 1% glucose. *GUT1–lacZ* expression was comparable to the expression observed on 2% glucose medium, indicating that full *GUT1–lacZ* expression was only possible in medium lacking glucose.

The pattern of *GUT1* expression was substantiated by northern blot analysis of *GUT1* expression on different carbon sources. *GUT1* was expressed on the non-fermentable carbon sources (glycerol and ethanol), whereas a low amount of *GUT1* transcript was detected in glucose-grown cells (Fig. 2).

A UAS$_{ADR1}$ is necessary for *GUT1–lacZ* expression

The transcriptional activator Adr1p has previously been reported to be required for *GUT1* expression during growth on glycerol (6). Furthermore, after a shift from glucose to glycerol medium, *GUT1–lacZ* expression was reduced ~7-fold in an *adr1* disruptant when compared with a *ADR1* wild-type strain (data not shown).

To further characterize *ADR1*-mediated *GUT1* activation, the 5′-non-coding sequence was analyzed for putative Adr1p binding sites. A putative Adr1p binding site (5′-TTGGAG-N$_{22}$-GTGGAG-3′) was localized at position –221 to –189 bp in the 5′-non-coding region. This UAS$_{ADR1}$ contains two GGAG motifs. However, unlike the palindromic UAS1, which is the target for Adr1p in the *ADH2* promoter (41), the GGAG motifs are non-palindromic and located as direct repeats. Another possible Adr1p binding site was found at –510 to –494 bp upstream for the translation initiation start. This UAS$_{ADR1}$ (5′-ATCTCC-N$_6$-TGGGAG-3′) has similarity to the UAS1 upstream of glucose-repressible alcohol dehydrogenase, *ADH2* (41). In order to test if Adr1p-mediated *GUT1* activation involves these potential Adr1p binding sites, point mutations were introduced in the GGAG motifs in the *GUT1–lacZ* plasmid (pMG29), either alone or in different combinations.

The most dramatic effect of mutations in a GGAG motif was observed when the distal GGAG element in UAS$_{ADR1}$ (–221 to –189 bp) was mutated (pMG38) (Fig. 3). In glycerol-grown cells transformed with pMG38, β-galactosidase activity was reduced by 75% when compared with cells transformed with a wild-type promoter construct (pMG29). Double mutations in both GGAG motifs (pMG65) reduced *GUT1–lacZ* expression by >80% on glycerol. The putative Adr1p binding site at position –510 to –494 bp only had a minor influence on *GUT1–lacZ* expression on glycerol, since β-galactosidase activity was reduced by not more than 20% in cells transformed with a *GUT1–lacZ* reporter plasmid with mutations in either a single (data not shown) or both of the GGAG motifs (pMG75). UAS$_{ADR1}$ (–510 to –494 bp) is located in the open reading frame (*GOS1*) upstream of *GUT1* at the 5′-end and it seems likely that this location will affect the functionality of the UAS element.

Interaction between Adr1p and UAS$_{ADR1}$ in the *GUT1* promoter

Protein binding between an Adr1 polypeptide and UAS$_{ADR1}$ (–221 to –189 bp) was tested by electrophoretic mobility shift assay. A 6×His tagged Adr1 polypeptide (amino acids 17–162) comprising the two zinc fingers from Adr1p was expressed in *E.coli*. When the Adr1 polypetide was present in the cell extract, two Adr1-dependent protein–DNA complexes could be observed (Fig. 4, lane 2), an abundant complex and a less abundant complex which migrates more slowly. Adr1p probably binds simultaneously as a monomer to a single half-site and as a dimer to both half-sites of its recognition motif. These Adr1p-specific band shifts disappeared when either protein extract from uninduced *E.coli* cells (lane 1) or no protein extract was used (lane 4). Furthermore, the Adr1p-dependent complexes were competed away by addition of a 100-fold (lane 3) molar excess of unlabeled oligonucleotide. The fastest

Table 2. *GUT1–lacZ* expression in glycerol or glucose medium in strains harboring *ino2*, *opi1* or *mig1 mig2* disruptions

Strain	Relevant genotype	*GUT1–lacZ* reporter plasmid[a]	β-Galactosidase activity	
			3% glycerol	2% glucose
Effect of disruptions in *OPI1* and *INO2*				
W303-1A	*INO2 OPI1*	pMG29 (wt)	2.20 (0.40)	0.03 (<0.01)
W303-1A	*INO2 OPI1*	pMG41 (ΔUAS_{INO})	1.04 (0.35)	0.02 (<0.01)
BRS2001	*ino2 OPI1*	pMG29 (wt)	1.65 (0.36)	0.02 (0.01)
BRS2005	*INO2 opi1*	pMG29 (wt)	No growth	0.63 (0.08)
BRS2005	*INO2 opi1*	pMG41 (ΔUAS_{INO})	No growth	0.09 (0.01)
Effect of disruptions in *MIG1* and *MIG2*				
CEN.PK133-5D	*MIG1 MIG2*	pMG29 (wt)	2.50 (0.50)	0.02 (<0.01)
T453	*mig1 MIG2*	pMG29 (wt)	2.45 (0.36)	0.08 (0.03)
B290	*mig1 mig2*	pMG29 (wt)	2.31 (0.40)	0.09 (<0.01)

The strains were transformed with the indicated *GUT1–lacZ* reporter constructs. Specific β-galactosidase activities are given in Miller units. Standard deviations of mean values are given in parentheses.
[a]Type of *GUT1* promoter indicated in parentheses; wt, wild-type promoter; ΔUAS_{INO}, with mutations in UAS_{INO}.

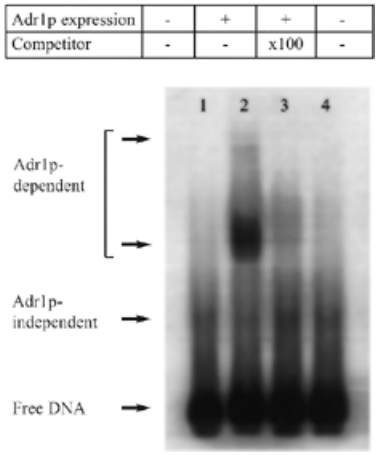

Figure 4. Binding of an Adr1 polypeptide to the UAS_{ADR1} (–221 to –189 bp) in the *GUT1* promoter region. As a probe for the electrophoretic mobility shift assay a 39 bp radiolabeled synthetic DNA fragment covering this *cis*-acting element was used. Two micrograms of *E.coli* protein extract was made from either uninduced or induced cells harboring an IPTG inducible plasmid, pQE31, which expresses amino acids 17–162 of Adr1p. As a competitor a 100-fold excess of unlabeled DNA fragment was used.

migrating complex is presumably binding of a single Adr1p to UAS_{ADR1}, whereas the slower migrating complex is binding of two Adr1 proteins, as has been observed for interactions between Adr1p and the UAS1 element in the *ADH2* promoter (13). Quantification of these Adr1p-dependent complexes showed that the upper complex represented 7% of the total radiolabeled DNA and the lower complex 43% (lane 2), indicating that Adr1p presumably prefers binding to one of the two possible binding sites in this region.

GUT1–lacZ expression is regulated by Ino2p and Ino4p

An UAS_{INO} (5′-CATGTGAAAA-3′) was found on the non-coding strand in the *GUT1* promoter region at position –319 to –309 bp. To study the importance of this UAS_{INO}, mutations were introduced into the *GUT1–lacZ* reporter plasmid (pMG29), resulting in plasmid pMG41, and β-galactosidase activity was measured in wild-type (W303-1A) cells transformed with pMG29 or pMG41. *GUT1–lacZ* expression decreased by ~50% in glycerol medium (Fig. 3), indicating that *GUT1–lacZ* expression is dependent on this UAS_{INO}.

Since UAS_{INO} is required for full induction on glycerol medium, the effects of mutations in a cognate *trans*-acting factor (Ino2p) were examined. In an *ino2* disruptant (BRS2001), *GUT1–lacZ* expression was reduced by ~25% during growth in glycerol medium, when compared with the parental strain (W303-1A) (Table 2). These measurements were performed in SC ura^- medium, containing 11 μM inositol, a concentration which has been shown to repress Ino2/Ino4p-regulated genes (33). In order to test if inositol also represses *GUT1–lacZ* expression, β-galactosidase activity was measured in W303-1A cells grown in I^-C^- (no inositol and no choline) and I^+C^+ (75 μM inositol and 1 mM choline) media with either glycerol or glucose. In glycerol-grown cultures, no difference in *GUT1–lacZ* expression was observed (data not shown). However, during growth on I^-C^- and I^+C^+ media with glucose as carbon source, a 3-fold difference in *GUT1–lacZ* expression was observed (0.03 Miller units on I^+C^+ versus 0.09 Miller units on I^-C^-). These results indicate that inositol and choline have a minor effect on *GUT1–lacZ* expression when cells grow on glucose.

An *INO2*/*INO4*-dependent protein complex assembles with UAS_{INO} in the *GUT1* promoter

To determine if Ino2p and Ino4p are able to bind the *GUT1* UAS_{INO}, an electrophoretic mobility shift assay with cell

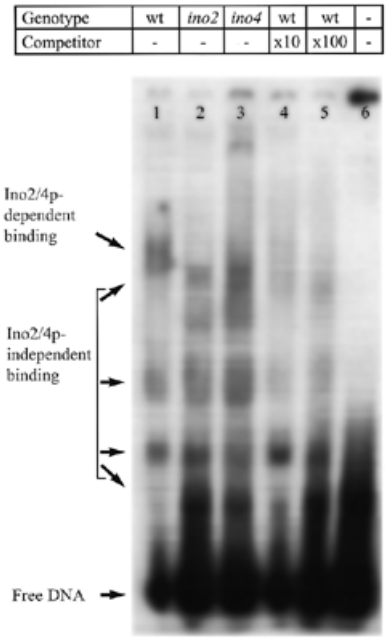

Figure 5. Binding of the Ino2/Ino4 protein complex to the UAS_{INO} in the *GUT1* promoter. As a probe for the EMSA a 31 bp radiolabeled double-stranded oligonucleotide covering this *cis*-acting element was used. Ten micrograms of yeast cell extract from W303-1A (*INO2 INO4*), BRS2001 (*ino2*) or BRS2004 (*ino4*) cells were used. As a competitor, either a 10- or 100-fold excess of unlabeled DNA fragment were used.

extracts from wild-type (W303-1A) cells and *ino2*- and *ino4*-disrupted (BRS2001 and BRS2004) cells was performed. The DNA template was ^{32}P-labeled double-stranded oligonucleotide containing UAS_{INO} flanked by 9 bp of promoter sequence. Cell extract from wild-type cells resulted in a specific shift (Fig. 5, lane 1), which disappeared on addition of either a 10- (lane 4) or 100-fold (lane 5) molar excess of unlabeled oligonucleotide. Using cell extracts from *ino2* or *ino4* disruptants, the upper band in the doublet observed with wild-type extract was not present (lanes 2 and 3). The lower shift in the observed *INO2*/*INO4*-dependent double shift was probably because of binding by a nonamer binding factor (NBF) to the *GUT1* UAS_{INO}. The non-characterized NBF was found to bind the UAS_{INO} from the inositol-1-phosphate synthase gene, *INO1* (20).

OPI1 is required for repression of *GUT1* expression

The observation that *GUT1–lacZ* expression on glycerol medium is activated through the *cis*-acting promoter element, UAS_{INO}, led us to test the influence of the regulatory protein Opi1p. Opi1p is a negative regulator of many, if not all, Ino2/Ino4p-regulated genes (18,25). Non-functional Opi1p results in derepression of Ino2p/Ino4p-regulated genes and the cells excrete inositol into the medium. As seen in Figure 1, *GUT1–lacZ* expression was shown to be repressed by glucose. In order to test if this glucose repression could be mediated by Opi1p, an *opi1* disrupted strain (BRS2005) was transformed with *GUT1–lacZ* reporter plasmids containing either wild-type promoter (pMG29) or a promoter without UAS_{INO} (pMG41). In glucose-grown cultures, β-galactosidase activity was found to be increased by a factor of ~20 (Table 2) in the *opi1* strain harboring pMG29. No further putative UAS_{INO} were found in the *GUT1* promoter region, which could explain the higher *GUT1–lacZ* expression found in the *opi1* strain harboring the *GUT1–lacZ* reporter construct (pMG41) when compared with the parental strain (W303-1A) harboring the same plasmid. Cells harboring an *opi1* disruption did not grow in medium with the non-fermentable carbon sources glycerol, ethanol or lactate, which made it impossible to measure *GUT1–lacZ* expression under derepressing conditions. This phenomenon has not been studied further.

INO2 expression is partially derepressed by inositol and choline on glycerol

Most of the genes regulated by the Ino2/Ino4 protein complex are repressed in the presence of inositol and choline (18). As seen in Table 2 and Figure 3, UAS_{INO} is necessary for full *GUT1–lacZ* expression on glycerol and an *INO2*/*INO4*-dependent protein complex is able to bind this *cis*-acting element (Fig. 5). Since *GUT1–lacZ* expression on glycerol is independent of the presence of inositol and choline, the expression of an integrated *INO2–cat* reporter construct [W303-1A(*INO2-cat*)] (26) was measured in glycerol medium with or without inositol and choline. Previous work has shown that *INO4* is constitutively expressed, whereas *INO2* expression is autoregulated in the absence of inositol and choline (26). Since the *INO2* promoter is one of the weakest promoters known in *S.cerevisiae* (26), a single copy integrated *INO2–cat* construct was used, because of the high sensitivity of the CAT assay (39).

During growth on glycerol and glucose without inositol and choline, approximately equal levels of *INO2–cat* expression were observed on these two carbon sources (Table 3). Addition of inositol gave repression of *INO2* on both carbon sources, but repression on glycerol medium was slightly lower than on glucose medium. When the same experiment was performed with addition of both inositol and choline, *INO2–cat* expression on glucose medium decreased further and more than on glycerol medium, thus the expression was 2.9-fold higher on glycerol than on glucose. Since *INO2* expression has been shown to be autoregulated by Ino2/Ino4p (26), it is likely that the partially repressed *INO2* expression is sufficient to activate *GUT1* on glycerol medium with inositol and choline.

Table 3. The effect of inositol and choline on *INO2* expression in media with either glycerol or glucose as carbon source

Inositol (75 μM)	Choline (1 mM)	CAT activity 3% glycerol	2% glucose	Fold difference glycerol/glucose
–	–	0.41 (0.12)	0.45 (0.05)	0.9
+	–	0.27 (0.01)	0.18 (0.01)	1.5
+	+	0.20 (0.02)	0.07 (0.01)	2.9

CAT activity was assayed in extracts of W303-1A cells containing a single copy of an *INO2–cat* reporter construct integrated in *GAL4* (26). Standard deviations of mean values are given in parentheses.

Glucose repression of *GUT1* is slightly relieved in *mig* mutants

The *GUT1* promoter includes a putative binding site for Mig1p (Cat4p, Ssn1p) (5′-AAAATGTGGGG-3′) located at position –82 to –72 bp in the *GUT1* promoter. This C_2H_2 zinc finger protein is one of the main factors in glucose repression (42,43). The putative URS_{MIG} element has a high degree of similarity to functional Mig1p binding sites from other glucose-repressed genes, such as *SUC2* and *GAL1*, as well as Mig1p binding sites in the upstream regions of pathway-specific activators such as *GAL4*, *HAP4* and *MAL63* (43,44).

Mig2p has high similarity to Mig1p and binds the same DNA motif (45). To address the question of the role of Mig1p and Mig2p on *GUT1* expression, mutations were introduced in the five guanines at the 3′-end of the URS_{MIG} element in a *GUT1–lacZ* plasmid (pMG37). On glucose medium, mutations in URS_{MIG} (pMG37) increased *GUT1–lacZ* expression 2-fold, whereas no change was observed in glycerol medium (Fig. 3). A similar result was obtained by measuring *GUT1–lacZ* expression in *mig1* (T453) and *mig1 mig2* (B290) strains transformed with a *GUT1–lacZ* reporter plasmid (pMG29) (Table 2). In these transformants, *GUT1–lacZ* expression increased 3- to 4-fold in glucose-grown *mig1* and *mig1 mig2* cells, when compared with the parental strain, CEN.PK113-5D.

DISCUSSION

The data presented in this paper demonstrate a carbon source transcriptional regulation of the glycerol kinase gene, *GUT1*, mediated by activation and repression systems. Growth on non-fermentable carbon sources derepresses *GUT1* expression, whereas expression is repressed by glucose, and also in the presence of glycerol (Fig. 1). These results support earlier data from Sprague and Cronan (5). By using microarrays to measure expression of all of the open reading frames in the *S.cerevisiae* genome during a time course in a glucose culture, DeRisi *et al.* (46) also found that *GUT1* expression increased when the glucose concentration was <0.2%.

The transcriptional activator Adr1p had earlier been reported to be required for *GUT1* expression (6). Our results support these data and show that *GUT1–lacZ* expression on glycerol medium is largely due to the left GGAG motif in UAS_{ADR1} (–221 to –189 bp). Mutations in this *cis*-acting element reduced expression by 75% during growth on glycerol (Fig. 3) and *in vitro* experiments showed that recombinant Adr1p was able to bind this UAS_{ADR1} almost exclusively as a monomer even in the presence of two possible GGAG binding sites (Fig. 4). However, an extensive study of Adr1p binding to the 22 bp palindromic sequence (UAS1) from glucose-repressible alcohol dehydrogenase, *ADH2*, showed that Adr1p binds as two independent monomers (13). For UAS1 it was furthermore shown that Adr1p could bind as a single monomer in the presence of only one GGAG binding site, but a single Adr1p was unable to activate a *CYC1* minimal promoter in a *CYC1–lacZ* reporter plasmid. The precise mechanism of *GUT1–lacZ* expression mediated by Adr1p through this UAS_{ADR1} is not clear, but several explanations are possible, such as: (i) in the right promoter context, Adr1p is able to activate as a single monomer; (ii) Adr1p interacts with one or several proteins, e.g. through the 506 N-terminal domain of the protein required for growth on glycerol (16); or (iii) Adr1p is involved in chromatin remodeling, such as described for *ADH2* (47), which could allow binding of other DNA binding proteins or change TATA box accessibility to the transcription machinery.

Different experiments showed that the basic helix–loop–helix transcription factors Ino2p and Ino4p are involved in *GUT1–lacZ* expression. During growth on glycerol, *GUT1–lacZ* expression decreased when either mutation was introduced in UAS_{INO} (Fig. 3) or when *INO2* was disrupted (Table 2). Furthermore, Ino2p/Ino4p is able to bind UAS_{INO} *in vitro* (Fig. 5). How inositol and choline mediate repression of Ino2p/Ino4p-regulated phospholipid genes is only partly understood. CDP-diacylglycerol synthase converts phosphatidic acid (PA) and CTP to CDP-diacylglycerol in the upper part of phospholipid biosynthesis. It has been shown that the mRNA levels of inositol-1-phosphate synthase, *INO1*, and phosphatidylserine synthase, *CHO1*, are regulated according to the CDP-diacylglycerol synthase activity in the cell (48,49). On decreasing CDP-diacylglycerol synthase activity, the PA pool and the amount of *INO1* and *CHO1* mRNA transcript is increased. The size of the PA pool has recently been suggested to mediate signals for repression/derepression of Ino2/Ino4p-regulated genes (18). Since PA is synthesized from glycerol-3-phosphate (17), it is possible that glycerol-grown cells have a higher PA pool than glucose-grown cells, which serves as an inducing signal to Opi1p and Ino2p/Ino4p. *INO2* expression is less repressed by inositol and choline when the cells use glycerol as carbon source instead of glucose (Table 3), which could explain the insensitivity of *GUT1* expression to inositol and choline during growth on glycerol. UAS_{INO} is located just before the very last codon in the *GOS1* gene, located upstream of *GUT1*, and it cannot be excluded that transcription of *GOS1* can affect the functionality of UAS_{INO}.

GUT1 repression on glucose medium mediated by *OPI1* is unexpected, because *OPI1* has until now only been described as a repressor of Ino2p/Ino4p-regulated genes in the presence of inositol. Our results indicate that Opi1p mediates repression of the *GUT1* promoter (Table 2). Opi1p contains both a leucine zipper and two polyglutamine stretches, which are often found in proteins with DNA binding properties and regulatory functions (25). Both elements are required for function of Opi1p (50), but it has until now not been reported to bind DNA and little is known about the molecular mechanisms used by Opi1p. Repression of structural genes required for phospholipid biosynthesis has also recently been shown to require the protein phosphatase Glc7p-Reg1p and the protein kinase Snf1p-Snf4p, which are components of the glucose repression system (27,28). When acting as a repressor of *GUT1* expression, Opi1p could possibly receive signals from components like Glc7p-Reg1p or Snf1p-Snf4p and mediate repression through Ino2p/Ino4p.

The zinc finger proteins Mig1p and Mig2p, which repress a large number of genes in the presence of glucose (51), play a minor role in glucose repression of *GUT1* (Table 2). One could speculate that the putative URS_{MIG} element is located too close to the open reading frame (–82 to –72 bp), however, functional URS_{MIG} elements are found at similar proximities to the open reading frame of the *GAL4* gene (42).

During growth on glycerol, ~90% of the expression seemed to be mediated through UAS_{INO} and the left GGAG motif in UAS_{ADR1} (–221 to –189 bp) (Fig. 3). It is not clear which

promoter element(s) is responsible for the remaining activation. A search did not result in additional candidates for regulatory elements, such as, for example, carbon source-responsive elements (CSRE), which have been shown to regulate some gluconeogenetic and glyoxylytic genes (52,53). *GUT1* expression has also been reported to be unaffected by deletion of the transcriptional co-repressor *TUP1* and by overexpression of the transcriptional activator *YAP1* (46).

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