

# Mechanism of Metabolic Control: Target of Rapamycin Signaling Links Nitrogen Quality to the Activity of the Rtg1 and Rtg3 Transcription Factors

Arash Komeili,<sup>\*‡</sup> Karen P. Wedaman,<sup>§</sup> Erin K. O'Shea,<sup>\*‡</sup> and Ted Powers<sup>§</sup>

<sup>\*</sup>Howard Hughes Medical Institute and <sup>‡</sup>Department of Biochemistry and Biophysics, University of California School of Medicine, San Francisco, California 94143; and <sup>§</sup>Section of Molecular and Cellular Biology, Division of Biological Sciences, University of California Davis, Davis, California 95616

**Abstract.** De novo biosynthesis of amino acids uses intermediates provided by the TCA cycle that must be replenished by anaplerotic reactions to maintain the respiratory competency of the cell. Genome-wide expression analyses in *Saccharomyces cerevisiae* reveal that many of the genes involved in these reactions are repressed in the presence of the preferred nitrogen sources glutamine or glutamate. Expression of these genes in media containing urea or ammonia as a sole nitrogen source requires the heterodimeric bZip transcription factors Rtg1 and Rtg3 and correlates with a redistribution of the Rtg1p/Rtg3 complex from a predominantly cytoplasmic to a predominantly nuclear location. Nuclear import of the complex requires the cytoplasmic protein Rtg2, a previously identified up-

stream regulator of Rtg1 and Rtg3, whereas export requires the importin- $\beta$ -family member Msn5. Remarkably, nuclear accumulation of Rtg1/Rtg3, as well as expression of their target genes, is induced by addition of rapamycin, a specific inhibitor of the target of rapamycin (TOR) kinases. We demonstrate further that Rtg3 is a phosphoprotein and that its phosphorylation state changes after rapamycin treatment. Taken together, these results demonstrate that target of rapamycin signaling regulates specific anaplerotic reactions by coupling nitrogen quality to the activity and subcellular localization of distinct transcription factors.

**Key words:** gene expression • metabolism • phosphorylation • rapamycin • signal transduction

## Introduction

Normal cell growth requires that cells adjust their metabolic activity according to nutrient availability and other environmental cues. Specialized signal transduction mechanisms exist that enable cells to perceive and integrate these cues in order to establish and/or maintain appropriate patterns of gene expression. Understanding how these pathways function is thus important for understanding both normal cellular behavior and the underlying basis of many human diseases, including cancer. One important signaling pathway used by all eukaryotic cells is the target of rapamycin (TOR)<sup>1</sup> kinase pathway. This pathway was discovered through the action of the antibiotic rapamycin, a potent inhibitor of T cell proliferation, which combines with the small immunophilin FKBP and targets the large, evolutionarily conserved TOR kinase (Thomas and Hall,

1997; Dennis et al., 1999). Rapamycin inhibits the growth of a wide variety of cell types and organisms, including the budding yeast, *Saccharomyces cerevisiae*. Yeast contains two TOR genes, *TOR1* and *TOR2*, and the products of both are inhibited by the rapamycin/FKBP complex (Heitman et al., 1991; Helliwell et al., 1994; Zheng et al., 1995). Treating yeast cells with rapamycin has several distinctive effects that mimic nutrient starvation, including inhibition of protein synthesis, cell cycle arrest at the G1/S boundary, onset of autophagy, and inhibition of ribosomal biogenesis (Heitman et al., 1991; Helliwell et al., 1994; Zheng et al., 1995; Barbet et al., 1996; Noda and Ohsumi, 1998; Zaragoza et al., 1998; Powers and Walter, 1999).

Recent studies have shown that TOR kinase activity is essential for the transcription of ribosomal RNA and ribosomal protein genes, as well as for the modulation of r-protein gene expression in response to changes in nutrient sources (Zaragoza et al., 1998; Powers and Walter, 1999). These results demonstrate that control of gene expression at the level of transcription represents an important branch of TOR signaling. This conclusion has been extended dramatically by results of recent microarray studies

Address correspondence to Ted Powers, Section of Molecular and Cellular Biology, Division of Biological Sciences, University of California Davis, Davis, CA 95616. Tel.: (530) 754-5052. Fax: (530) 752-3085. E-mail: tpowers@ucdavis.edu

<sup>1</sup>Abbreviations used in this paper: GFP, green fluorescent protein; HA, hemagglutinin; MD, minimal dextrose; ORF, open reading frame; SCD, synthetic complete dextrose; TOR, target of rapamycin.

where the expression of several hundred genes has been shown to be affected when TOR function is inhibited by rapamycin (Cardenas et al., 1999; Hardwick et al., 1999). In addition to genes involved in protein biosynthesis, many of the affected genes are involved in the glycolytic pathway, the TCA cycle, and nitrogen metabolism. Thus, one important role of TOR appears to be the coordination of the transcription of genes involved in several distinct nutrient-responsive cellular pathways.

One of the most striking sets of genes affected by rapamycin treatment is composed of genes involved in the use of different sources of assimilable nitrogen (Cardenas et al., 1999; Hardwick et al., 1999; C. Kao, T. Powers, P. Walter, G. Crabtree, and P. Brown, unpublished results). For example, a sharp decrease is observed in the expression of genes involved in the uptake and metabolism of preferred nitrogen sources, including glutamine and asparagine. In contrast, a corresponding increase is observed in the expression of genes involved in the uptake and use of alternative nitrogen sources, including urea, proline, and allantoin (a product of purine catabolism). These results establish a link between TOR signaling and nitrogen metabolism and suggest that one crucial role of this pathway is to couple nitrogen availability to continued cell growth. Moreover, they are consistent with results from studies of mammalian cells that demonstrate that TOR signaling is influenced by amino acid availability (Iiboshi et al., 1999b). Taken together, these results are particularly relevant given that levels of preferred nitrogen sources in human cells (e.g., glutamine and asparagine) play an important role in the progression of several diseases, including childhood acute lymphoblastic leukemia and these levels may be regulated, at least in part, by TOR (Iiboshi et al., 1999a).

The molecular mechanism by which TOR controls the expression of several genes involved in nitrogen metabolism, including *GLN1*, *MEP2*, and *GAPI*, has been shown recently to involve regulated changes in the subcellular localization of the Gln3 transcription factor (Beck and Hall, 1999). Thus, in the presence of preferred sources of nitrogen, Gln3 is sequestered in the cytoplasm by association with the Ure2 protein, a previously identified negative regulator of Gln3 function. TOR promotes formation of a Gln3-Ure2 complex by a mechanism that involves inhibition of the Sit4 phosphatase and that requires the TOR effector protein Tap42 (Di Como and Arndt, 1996; Beck and Hall, 1999; Jiang and Broach, 1999). Treating yeast cells with rapamycin or, alternatively, introducing them into nitrogen-poor media, causes Gln3 to become dephosphorylated and to dissociate from Ure2, where it then moves into the nucleus to activate its target genes (Beck and Hall, 1999). Two additional studies suggest that TOR-dependent changes in the phosphorylation of Ure2 are also important for regulating the stability of the Gln3-Ure2 complex (Cardenas et al., 1999; Hardwick et al., 1999). Rapamycin treatment stimulates nuclear accumulation of two other transcriptional activators, Msn2 and Msn4, both of which respond to different sources of cellular stress, including carbon source limitation (Beck and Hall, 1999). Thus, it is likely that additional nutrient-responsive transcription factors will also turn out to be regulated by TOR.

In addition to permeases and degradative enzymes required for the use of specific nitrogen sources, distinct

pathways involved in carbon metabolism are also responsive to nitrogen availability. For example, it has been observed that several genes encoding enzymes involved in the TCA and glyoxylate cycles are required for glutamate prototrophy (Ogur et al., 1964, 1965; Kim et al., 1986; Gangloff et al., 1990). These genes include *ACO1*, which encodes mitochondrial aconitase, and *CIT1* and *CIT2*, which encode mitochondrial and peroxisomal forms of citrate synthase, respectively (Ogur et al., 1964; Kim et al., 1986; Rosenkrantz et al., 1986; Gangloff et al., 1990). Moreover, recent studies demonstrate that expression of these genes is subject to repression by glutamate (Liu and Butow, 1999). These results reflect the fact that in addition to providing reduced NADH and FADH<sub>2</sub> for mitochondrial respiration, the TCA cycle is also the source of many biosynthetic intermediates, including  $\alpha$ -ketoglutarate, the primary precursor to glutamate (Stryer, 1995). Thus, in the absence of exogenously supplied glutamate (or glutamine), these intermediates must be replaced through additional anaplerotic reactions to keep the TCA cycle operational and to maintain the respiratory competency of the cell. Recent studies have demonstrated that glutamate-mediated inhibition of *ACO1*, *CIT1*, and *CIT2* involves regulation of the heterodimeric bHLH/Zip transcription factors Rtg1 and Rtg3 (Liu and Butow, 1999). The mechanism by which these transcription factors are regulated according to nitrogen availability, however, is not well understood.

Studies of both prokaryotic and eukaryotic cells emphasize glutamate and glutamine as important regulators of nitrogen metabolism (reviewed by Magasanik, 1992; Merrick and Edwards, 1995; Marzluf, 1997). Both are used in the biosynthesis of other amino acids and, as discussed above, can be readily converted to  $\alpha$ -ketoglutarate for use in the TCA cycle. Glutamine is also an immediate precursor for the biosynthesis of nucleotides and other nitrogen-containing molecules, including NAD<sup>+</sup>, and thus represents a primary means by which nitrogen is assimilated into cellular material. Not surprisingly, cells have evolved elaborate mechanisms to sense the intracellular levels of these amino acids and to use this information to regulate their uptake and/or synthesis. Studies of enteric bacteria have revealed a complex signaling pathway involving a two-component regulatory system that couples intracellular levels of glutamine to changes in gene expression (reviewed by Merrick and Edwards, 1995). Whether TOR signaling responds specifically to intracellular levels of glutamine and/or glutamate in eukaryotic cells is presently unknown.

We are interested in understanding further both the scope and the mechanisms by which gene expression is modulated according to nitrogen availability in yeast. Toward this end, we have explored a novel use of genome-wide expression analysis by identifying genes that are expressed differentially when yeast cells are grown in the presence of two defined nitrogen sources, the primary source glutamine versus an alternative source, urea. We find that a surprisingly small number of genes (<40) show significant differences in their levels of expression under these two conditions, where each identified gene is either induced or repressed by glutamine. In addition to Gln3-dependent target genes, one of the most concise sets of genes subject to glutamine-mediated repression includes metabolic genes regulated by the transcription factors

Table I. Strains of *S. cerevisiae* Used in this Study

S288c	<i>Mata gal2 mal</i>	Botstein Lab strain collection*
DBY7286	<i>Mata ura3</i>	Botstein Lab strain collection*
PYL037	same as DBY7286, except for <i>rtg1Δ::URA3</i>	This work
PLY039	same as DBY7286, except for <i>rtg3Δ::URA3</i>	This work
DBY8943	<i>Mata his3Δ::hisG</i>	Botstein Lab strain collection*
PLY047	same as DBY8943, except for <i>RTG1-3HA:HIS3MX6</i>	This work
PLY050	same as DBY8943, except for <i>RTG3-3HA:HIS3MX6</i>	This work
PLY089	same as DBY8943, except for <i>RTG2-3HA:HIS3MX6</i>	This work
JK9-3da	<i>Mata leu2-3, 112 ura3-52 rme1 trp1 his4</i>	Barbet et al., 1996
JH11-1c	same as JK9-3da, except for <i>TOR1-1</i>	Barbet et al., 1996
PLY079	same as EY0733, except for <i>TOR1-1</i> , pRTG1-GFP	This work
PLY083	same as EY0735, except for <i>TOR1-1</i> , pRTG3-GFP	This work
K699	<i>Mata ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+</i>	Nasmyth et al., 1990
EY0733	same as K699, except for <i>rtg1Δ::TRP1</i>	This work
EY0734	same as K699, except for <i>rtg2Δ::TRP1</i>	This work
EY0735	same as K699, except for <i>rtg3Δ::TRP1</i>	This work
EY0736	same as K699, except for <i>msn5Δ::HIS3</i>	This work
EY0744	same as K699, except for <i>rtg2Δ::TRP1 msn5Δ::HIS3</i>	This work

\*See Materials and Methods.

Rtg1 and Rtg3. We demonstrate that, like Gln3, Rtg1 and Rtg3 are regulated by changes in their subcellular localization according to available nitrogen and, moreover, that the TOR kinase pathway plays an essential role in this regulation. Our data further suggest that glutamine-responsive transcriptional modulation defines a distinct branch of TOR signaling in yeast.

## Materials and Methods

### Strains, Media, and General Methods

All strains of *S. cerevisiae* used in this study are listed in Table I. The following culture media was used: YPD (1% yeast extract, 2% peptone, 2% dextrose); minimal dextrose (MD) (0.8% yeast nitrogen base without amino acids and ammonium sulfate, pH 5.5, 2% dextrose); synthetic complete dextrose (SCD) (0.7% yeast nitrogen base without amino acids, pH 5.5, 2% dextrose). MD media contained in addition one or more of the following nitrogen sources: glutamine, glutamate, ammonia, or urea, as indicated in the text, each at 0.2% final concentration. To supplement the auxotrophic requirements of strains used for the fluorescence microscopy experiment presented in Fig. 4 (below), required amino acids, adenine, and uracil were added to MD media at concentrations described by Sherman (1991). SCD media was also supplemented with appropriate amino acids, adenine and uracil as described by Sherman (1991). Yeast cultures were grown at 30°C for all experiments. Yeast transformations were performed using a DMSO-enhanced lithium acetate procedure (Hill et al., 1991). Rapamycin (Sigma-Aldrich) was dissolved in DMSO and added to a final concentration of 0.2 μg/ml unless stated otherwise.

### Gene Expression Analysis Using cDNA Microarrays

Strain S288c was grown with vigorous shaking to 0.5 OD<sub>600</sub>/ml in 1 liter of MD media containing appropriate nitrogen sources, as indicated in the text. Cells were immediately harvested by centrifugation, flash frozen in liquid nitrogen, and stored at -80°C. Relative mRNA levels were determined by hybridizing fluorescently labeled cDNAs to microarrays containing cDNAs representing virtually every yeast open reading frame (DeRisi et al., 1997; Lashkari et al., 1997). Arrays were produced under the auspices of J. Derisi at UCSF by a consortium of laboratories affiliated with the Department of Biochemistry and Biophysics. Primers for amplification of the yeast genome were purchased from Research Genetics and were provided by the laboratory of P. Walter. Arrays were scanned using a GenePix 4000a Microarray Scanner (Axon Instruments, Inc.) and analyzed using software provided by the manufacturer. Data were also analyzed using software available through the web site affiliated with the laboratories of P. Brown and D. Botstein at Stanford University (Stanford,

CA; <http://rana.Stanford.EDU/software/>). Scatterplots shown in Fig. 2 (below) were constructed using Excel software (Microsoft Corp.). The complete data set for the nitrogen source experiments presented here will be made available upon request.

### Northern Blots

Northern-blot analysis was performed as described previously (Powers and Walter, 1999). DNA probes were generated by PCR using genomic DNA from strain S288c as template and specific primers (purchased from Research Genetics) for individual genes (open reading frame, ORF, names are listed in Table II). Quantitation of blots was performed using a STORM 860 imaging system (Molecular Dynamics) and analyzed using software provided by the manufacturer.

### Plasmid Construction

Green fluorescent protein (GFP)-tagged plasmids were constructed by PCR amplification of the promoter and ORF of *RTG1*, *RTG2*, and *RTG3*. Primers were designed such that 500 base pairs of upstream promoter region and the entire ORF of each gene were amplified. Each 5' upstream primer contained an XhoI restriction endonuclease site and each 3' downstream primer contained an EcoRI restriction endonuclease site immediately following the stop codon. After digestion with XhoI and EcoRI, each fragment was introduced in the XhoI and EcoRI sites of pRS316-GFP, which contains GFP<sup>S65T</sup> (Kaffman et al., 1998b). The resulting plasmids, pRtg1-GFP, pRtg2-GFP, and pRtg3-GFP produced versions of Rtg1-Rtg3 that contained GFP<sup>S65T</sup> fused to their COOH termini. Plasmid pRtg1-GFP3, which contains *RTG1* fused in frame with three tandem copies of GFP<sup>S65T</sup>, was constructed by replacing the promoter and coding region of pPHO4-GFP<sub>3</sub> (Kaffman et al., 1998b) with the corresponding promoter and coding region of *RTG1*. pRtg3-zz was constructed by replacing the GFP in pRtg3-GFP with two Protein A z domains (zz) as described (Kaffman et al., 1998b). All plasmid-expressed, tagged genes were tested for their ability to complement the null phenotype of the appropriate deletion strains.

### Construction of Yeast Strains

Strains derived from DBY7286 that were deleted for *RTG1* or *RTG3* were constructed using standard gene replacement techniques (Rothstein, 1991). The entire coding regions of both genes were replaced with the *URA3* gene from pRS306 (Sikorski and Heiter, 1989). These strains were used for the experiments presented in Figs. 3 and 6 (below).

Strains derived from DBY8943 that produced versions of Rtg1-Rtg3 tagged at their COOH termini with three copies of the hemagglutinin (HA) epitope were constructed using the PCR-based method described by Brachmann et al. (1998) and Longtine et al. (1998). As template for PCR, we used the plasmid pFA6a-3HA-HIS3MX6 that contained the *Schizosaccharomyces pombe* HIS3 homologue (Longtine et al., 1998). The

Table II. Summary of Nitrogen-regulated Genes Identified by Microarray Analysis

Functional class	ORF	Gene	Description	Expression ratio*	
Genes preferentially expressed in glutamine					
Transporters	YDR046C	<i>BAP3</i>	Amino acid permease	12.5	
	YBR068C	<i>BAP2</i>	Amino acid permease	17.4	
	YCL025C	<i>AGP1</i>	Amino acid permease	12.3	
	YBR069C	<i>VAP1</i>	Amino acid permease	3.5	
	YOL020W	<i>TAT2</i>	Tryptophan permease	3.4	
	YDR508C	<i>GNP1</i>	Glutamine permease	3.1	
	YGR055W	<i>MUP1</i>	Methionine permease	10.6	
	YER064C	Unknown	Putative small molecule transporter	3.3	
	YNL065W	Unknown	Putative small molecule transporter	3.2	
	Miscellaneous	YNL160W	<i>YGP1</i>	Stationary phase secreted glycoprotein	9.4
YCR021C		<i>HSP30</i>	Plasma membrane heat shock protein	4.6	
YFL014W		<i>HSP12</i>	Glucose and lipid heat shock protein	3.8	
Genes preferentially expressed in urea					
<i>DAL</i> cluster group	YIR027C	<i>DAL1</i>	Allantoinase	14.2	
	YIR029W	<i>DAL2</i>	Allantoinase	12.7	
	YIR032C	<i>DAL3</i>	Ureidoglycolate hydrolase	5.3	
	YIR028W	<i>DAL4</i>	Allantoin permease	18.8	
	YJR152W	<i>DAL5</i>	Allantoate and ureidosuccinate permease	17.8	
	YIR031C	<i>DAL7</i>	Glyoxysomal malate synthase	34.4	
	YKR034W	<i>DAL80</i>	Repressor of <i>GLN3</i> function	4.3	
	Transporters	YKR039W	<i>GAP1</i>	General amino acid permease	5.3
		YNL142W	<i>MEP2</i>	Ammonia permease	17.3
		YBR294W	<i>SUL1</i>	Sulfate transport protein	4.2
YHL016C		<i>DUR3</i>	Urea permease	35.9	
YLR348C		<i>DIC1</i>	Mitochondrial dicarboxylate carrier	3.3	
Metabolism/Enzymes	YPL265W	<i>DIP5</i>	Dicarboxylic amino acid permease	7.5	
	YBR208C	<i>DUR1,2</i>	Urea amidolyase	5.4	
	YLR142W	<i>PUT1</i>	Proline oxidase	10.6	
	YPR035W	<i>GLN1</i>	Glutamine synthetase	6.9	
	YNL117W	<i>MLS1</i>	Malate synthase 1	4.8	
	YGL062W	<i>PYC1</i>	Pyruvate carboxylase 1	7.5	
	YLR304C	<i>ACO1</i>	Aconitate hydratase	9.5	
	YNL037C	<i>IDH1</i>	Isocitrate dehydrogenase 1	7.7	
	YOR136W	<i>IDH2</i>	Isocitrate dehydrogenase 2	8.4	
	YCR005C	<i>CIT2</i>	Peroxisomal citrate synthase	10.5	
YEL071W	<i>DLD3</i>	D-Lactate dehydrogenase 3	9.1		

Prototrophic strain S288c was grown to 0.5 OD<sub>600</sub>/ml in 1 liter minimal dextrose media containing 0.2% glutamine or 0.2% urea as sole nitrogen sources. PolyA mRNA was isolated and used to prepare cDNAs that were fluorescently labeled with either Cy3 or Cy5 fluorescent dyes. Samples were mixed and applied to DNA microarrays containing >6,200 individual yeast genes and analyzed using a microarray laser reader. Shown are the results of three independent experiments. In two of these experiments, each sample was labeled with Cy3 as well as Cy5, generating a total of five independent microarrays used for analysis. Listed genes displayed a mean difference ratio of 3.0 or greater in each of the five microarrays. Several genes known from previous studies to be regulated by glutamine did not meet these strict criteria, including *PUT4*, *ARG3*, *CAR1*, and *ASP3,4*. Thus, this list is likely to represent an underestimate of the total number of glutamine-regulated genes. Gene names and descriptions were obtained from the SGD (<http://genome-www.stanford.edu/Saccharomyces>) and YPD (<http://www.proteome.com/databases/index>) databases.

\*Genes preferentially expressed in glutamine (glutamine vs. urea), genes preferentially expressed in urea (urea versus glutamine).

tagged genes were determined to be functional based on the normal growth of each resulting strain, PLY047, PLY050, and PLY089, on MD–ammonia and –urea agar plates. These strains were used for the experiment presented in Fig. 9 A (below).

Strains derived from K699 that were deleted for *RTG1*, *RTG2*, or *RTG3* were made using the same PCR-based gene disruption technique described above, using the *TRP1* gene of *Candida glabrata* as a selectable marker (Kitada et al., 1995). Primers used for PCR possessed 40 bases of homology that corresponded to the 5' and 3' ends of the open reading frame of each target gene. These strains, EY0733, EY0734, and EY0735, were transformed with an appropriate GFP fusion plasmid, described above, and used for the fluorescence microscopy experiments presented in Figs. 4, 5 A, and 7 (below).

*MSN5* was deleted from K699 using a *HIS3*-marked disruption vector, as described previously (Kaffman et al., 1998a). The *rtg2Δ msn5Δ* strain was made by mating the *rtg2Δ* and *msn5Δ* strains described above and selecting *TRP*<sup>+</sup> *HIS*<sup>+</sup> segregants after sporulation and tetrad dissection. The resulting *msn5Δ* and *rtg2Δ msn5Δ* strains, EY0736 and EY0744, respectively, were used for experiments presented in Fig. 8 (below).

Strains containing the *TOR1-1* allele combined with either *rtg1Δ*/pRTG1-GFP or *rtg3Δ*/pRTG3-GFP were constructed using the following approach. Strains EY0733 and EY0735 were transformed with pRTG1-GFP or pRTG3-GFP, respectively. The resulting transformants were mated to strain JH11-1c and diploids were selected by their ability to grow on SCD agar plates lacking both adenine and uracil. After sporulation, *TRP*<sup>+</sup>, *URA*<sup>+</sup> segregants were isolated and tested for their ability to grow on plates containing 0.2 μg/ml rapamycin. The resulting selected strains, PLY079 and PLY083, were used for the experiment shown in Fig. 5 B (below).

All wild-type parental strains used for construction of the above strains were examined by Northern blot analysis to confirm that expression of the *RTG*-dependent target genes *CIT2* and *DLD3* was (a) repressed by preferred nitrogen sources, and (b) induced by rapamycin treatment.

### Fluorescence Microscopy

For all microscopy experiments, cells were freshly transformed with plasmids that expressed appropriate GFP-fusion proteins. Cells were first grown overnight in SCD media that lacked uracil to select for plasmid

maintenance. To lower the background auto fluorescence of the parent strain, additional adenine and tryptophan were added to a final concentration of 0.005%. Cells were then diluted to 0.005 OD<sub>600</sub>/ml in media appropriate for each experiment, as indicated in the text, and were examined directly by fluorescence microscopy when they reached 0.5 OD<sub>600</sub>/ml. Rapamycin was added to a final concentration of 1.0 µg/ml for microscopy experiments presented in the figures. Identical results were also obtained at the lower rapamycin concentration of 0.2 µg/ml. All images documenting GFP localization were collected on an microscope with a 100× objective (BX60; Olympus) and recorded with a CCD camera (Photometrics) using identical settings for each experiment and an average exposure time of 1.0–1.5 s.

### Preparation of Cell Extracts and Western Blot Analysis

For detection of Rtg1-Rtg3 by immunoblotting, cells were grown in appropriate media, as described in the text, to 0.5 OD<sub>600</sub>/ml, treated with rapamycin where indicated, and harvested directly. Extracts were prepared as described previously (Kaffman et al., 1998b). As Rtg3 proved to be relatively unstable in cell extracts, even in the presence of protease inhibitors, fresh extracts were prepared for each experiment and were never frozen. To detect Rtg1-HA<sub>3</sub>, Rtg2-HA<sub>3</sub>, and Rtg3-HA<sub>3</sub>, 1–2 mg of extract was separated by SDS-PAGE as described (Kaffman et al., 1998b). Anti-HA Western blots were performed using affinity-purified 12CA5 antibodies (Babco). Rtg3-zz was immunoprecipitated using IgG-Sepharose beads as described (Amersham Pharmacia Biotech). For phosphatase experiments, immunoprecipitated Rtg3-zz was washed with IgG buffer and lambda phosphatase buffer (New England Biolabs, Inc.). Samples were split in half and beads were resuspended in 100 µl of lambda phosphatase buffer with 2 mM MnCl<sub>2</sub>. Approximately 800 U of lambda protein phosphatase were added to one of the two samples and the reactions were incubated for 30–45 min at 30°C. Samples were eluted by boiling the beads in 2× SDS sample buffer for 3 min and analyzed using anti-zz rabbit polyclonal antisera as described (Kaffman et al., 1998b).

## Results

### Identification of Nitrogen-regulated Genes

We used genome-wide gene expression analysis to compare mRNA levels following the growth of yeast cells in the presence of two distinct sources of assimilable nitrogen, glutamine and urea. Whereas glutamine is used directly in the biosynthesis of several nitrogen-containing compounds, urea must first be degraded, via a bifunctional enzyme encoded by the *DUR1,2* gene, to ammonia and carbon dioxide before the ammonium ion can be incorporated into glutamate and glutamine (Sumrada and Cooper, 1982). We chose urea in part because we observed that the prototrophic strain S288c grew with a similar doubling time of ~110 min in minimal dextrose media containing either glutamine (MD-glutamine) or urea (MD-urea) as a sole source of nitrogen. This was in contrast to several other alternative nitrogen sources that we tested, including arginine and proline, which resulted in reduced growth rates (data not shown). Thus we reasoned that any observed differences in gene expression would be restricted to the use of glutamine and urea as nitrogen sources, rather than secondary effects due to differences in growth rate. Accordingly, S288c was grown to mid-log phase in MD-glutamine or MD-urea and PolyA mRNA was isolated. Fluorescently labeled cDNAs were then prepared and applied to DNA microarrays that contained nearly every yeast open reading frame (DeRisi et al., 1997; Lashkari et al., 1997).

Of the more than 6,200 genes examined, a surprisingly small number (<40) displayed differences in expression threefold or greater under these two nitrogen conditions;

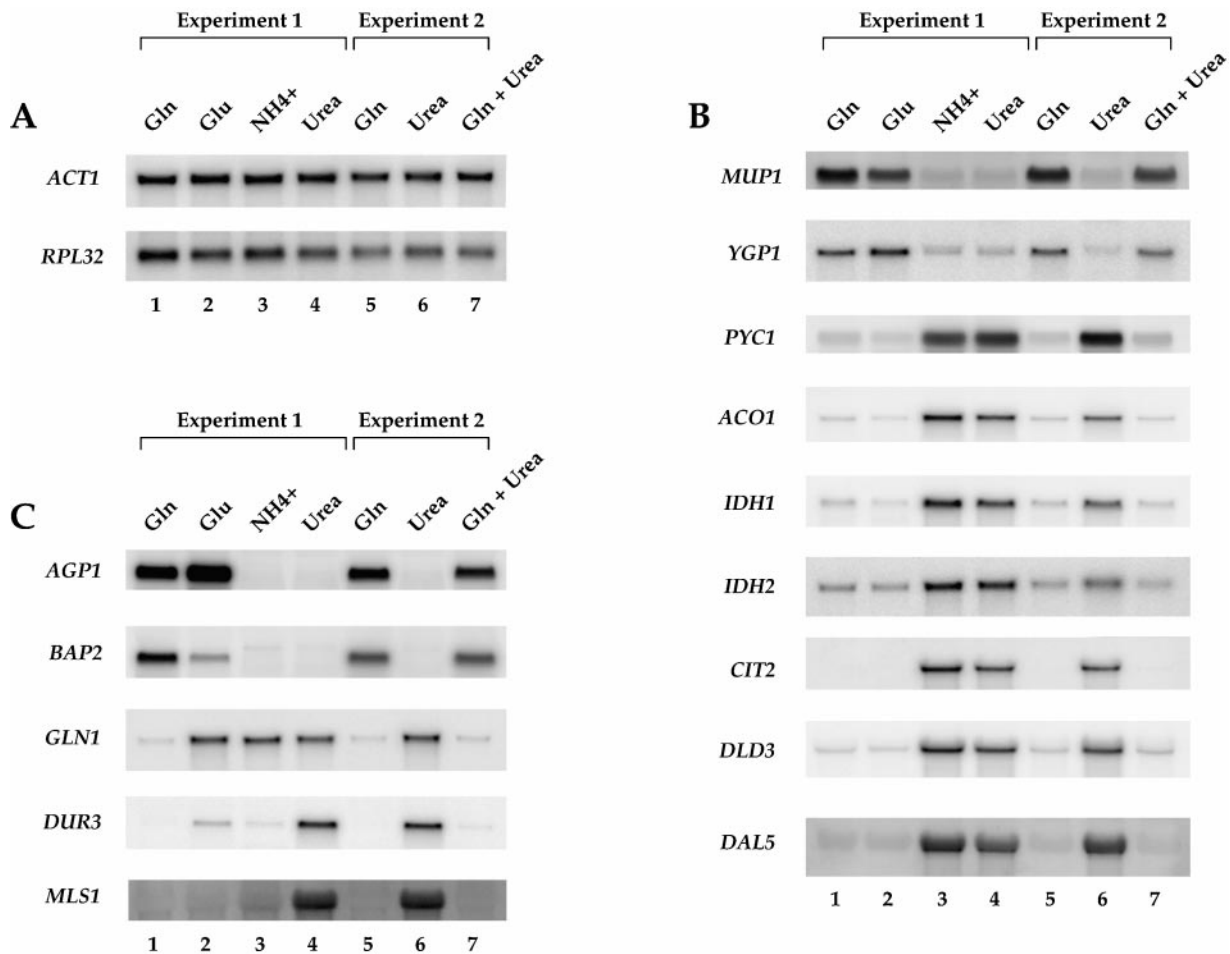
12 were expressed preferentially in MD-glutamine and 24 were expressed preferentially in MD-urea (Table II). We confirmed these results for a representative number of genes directly by Northern blot analysis (Fig. 1). The majority of genes that were expressed better in MD-glutamine encoded permeases specific for amino acids associated with rich nutrient conditions, including *GNPI* and *TAT2*, which encode high affinity permeases specific for glutamine and tryptophan, respectively (Zhu et al., 1996; Beck et al., 1999). In contrast, genes expressed preferentially in MD-urea could be grouped into one of three general classes: (a) transport permeases specific for poor nitrogen conditions, including *DUR3*, which encodes urea permease; (b) the majority of the *DAL* genes, which are involved in the uptake and catabolism of allantoin (Cooper et al., 1979; Cox et al., 1999); and (c) metabolic enzymes, including several associated with the citric acid and glyoxylate cycles (Table II). Given the relatively small number of genes identified in this experiment, these results suggest that a limited number of regulatory pathways are likely to be involved in the differential use of these two nitrogen sources.

To extend the above results, we determined the relative expression of a representative number of genes following the growth of cells in media containing one of two other preferred nitrogen sources, glutamate and ammonia. In general, the pattern of expression produced by cells in MD-glutamate was similar to that produced in MD-glutamine, particularly for the genes involved in the TCA and glyoxylate cycles (Fig. 1 B, compare lanes 1 and 2). Several differences were also observed, however. For example, *GLNI*, which encodes glutamine synthetase, was expressed in MD-glutamate but not in MD-glutamine (Fig. 1 C, compare lanes 1 and 2). This result demonstrates the extreme selectivity in gene expression that exists according to the precise nitrogen source provided (Magasanik, 1992).

Very few differences in gene expression were observed when ammonia and urea were compared, one of the most notable being *DUR3* (Fig. 1 C, compare lanes 3 and 4). Indeed, microarray analysis revealed that <10 genes were expressed differently when cells were grown in media containing each of these two nitrogen sources (T. Powers, unpublished results). These results were somewhat surprising given that ammonia is known to display many of the same regulatory properties as glutamine and glutamate (Magasanik, 1992). One likely explanation for this result is that some strains of *S. cerevisiae* harbor mutations that affect ammonia-dependent regulation of certain genes (Courchesne and Magasanik, 1983). The precise molecular basis for this observation has yet to be clarified.

### Demonstration of Glutamine as a Global Regulator of Gene Activity

The results of the above microarray experiment revealed the scope of genes whose expression differed significantly in the presence of glutamine versus urea. This experiment could not distinguish, however, whether these differences resulted from the stimulation or repression of gene activity by either nitrogen source. We reasoned that we could address this issue using microarrays by pair-wise compari-



**Figure 1.** Examples of differences in gene expression during growth of yeast cells in the presence of different sources of assimilable nitrogen. Strain S288c was grown to mid-log phase in MD media containing the indicated nitrogen sources. Total mRNA was isolated and Northern blot analysis was performed, probing for the specified mRNAs. (A) Control transcripts showing no significant differences under the conditions tested. (B) Transcripts displaying similar levels of expression in MD-glutamine and MD-glutamate. (C) Transcripts displaying more complex patterns of expression.

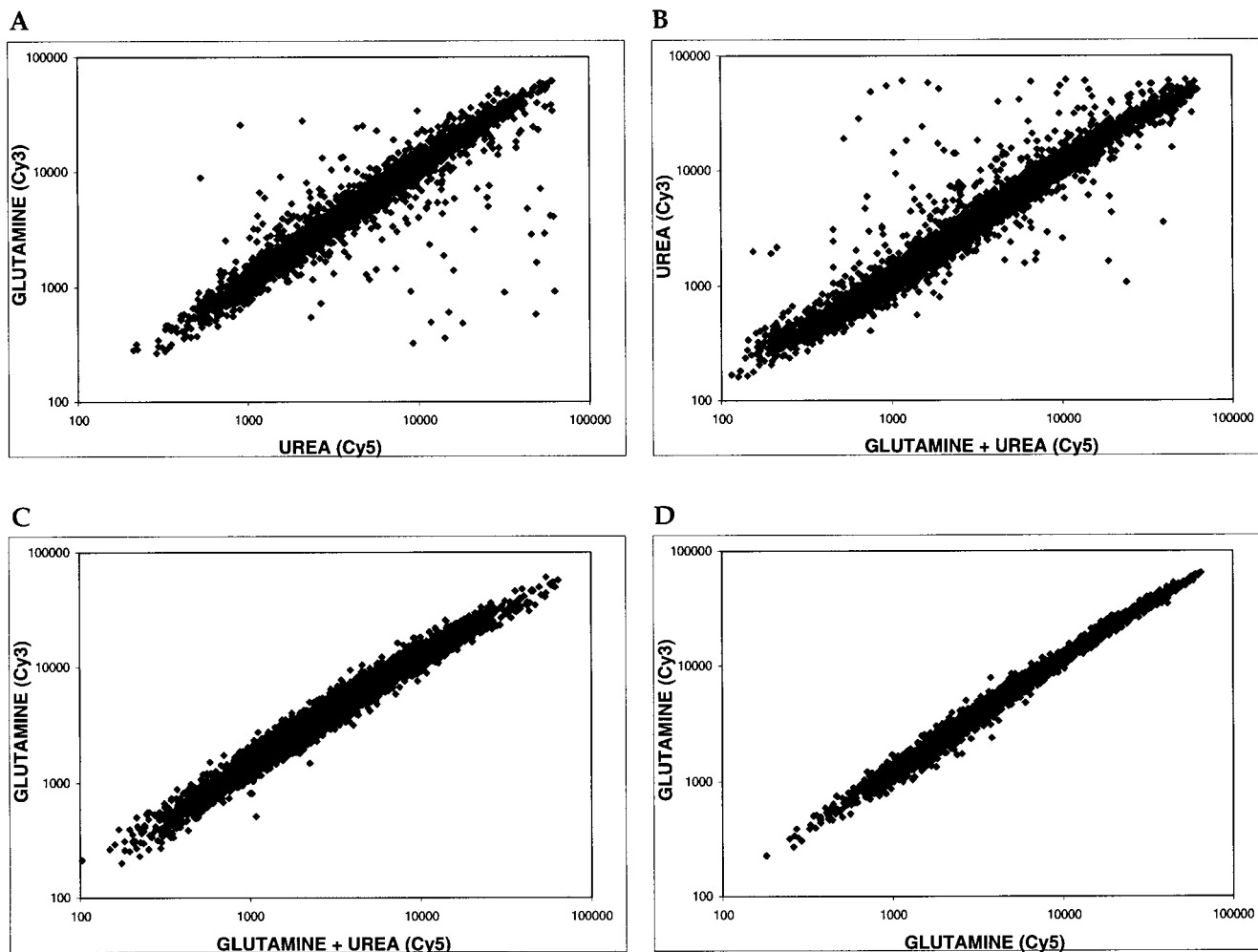
sons of cultures grown in the presence of one versus both nitrogen sources. The logic here was that a given gene should display the same level of expression (i.e., have a ratio  $\sim 1.0$ ) if the activating (or repressing) nitrogen source was present in the two samples being compared. Accordingly, we compared mRNA levels from cells grown in the following media: MD-glutamine, MD-urea, and MD-glutamine+urea. The results of this experiment are shown in the form of scatter plots in Fig. 2.

When mRNA levels from cells grown in MD-glutamine and MD-urea were compared, most genes appeared as points along a diagonal with a slope of  $\sim 1.0$  and corresponded to genes expressed similarly under the two conditions (Fig. 2 A). A characteristic number of points fell both above and below this diagonal and corresponded to genes (reported in Table II) that were preferentially expressed in glutamine or urea, respectively (Fig. 2 A). This pattern of expression was remarkably similar when cells grown in MD-urea and MD-glutamine+urea were compared, indicating that the presence of urea in both cultures did not significantly change the relative expression of any gene (Fig. 2, compare A and B; note that the plot in B ap-

pears as the reciprocal of the plot in A due to the arrangement of axes). In dramatic contrast, when MD-glutamine and MD-glutamine+urea samples were compared, essentially all points collapsed onto the diagonal, demonstrating the dramatic effect by glutamine on gene expression (Fig. 1 C). From these results, we conclude that essentially all differences in gene expression observed in these experiments result from glutamine acting as both an activator and a repressor of gene activity. This conclusion was confirmed for a number of representative genes by Northern blotting (Fig. 1, lanes 5–7) and is consistent with the demonstrated role of glutamine as an important regulator of nitrogen-dependent gene expression (Magasanik, 1992).

#### ***RTG-dependent Gene Expression: An Interface between Carbon and Nitrogen Metabolism***

Many of the differences in gene expression observed in the preceding experiments were likely to reflect altered metabolic needs as cells use distinct nitrogen sources. For example, de novo biosynthesis of amino acids requires intermediates provided by the TCA cycle, primarily oxaloacetate and  $\alpha$ -ketoglutarate, that must be replaced



**Figure 2.** Glutamine is both a global activator and repressor of gene expression. Scatter plots show pairwise comparisons of gene expression profiles of S288c cells grown in the presence of glutamine, urea, or glutamine + urea. (A) MD-glutamine versus MD-urea. (B) MD-urea versus MD-glutamine + urea. (C) MD-glutamine versus MD-glutamine + urea. (D) Control experiment comparing MD-glutamine with itself. For each plot, the x axis depicts cDNA samples labeled with Cy5 dye and the y axis depicts samples labeled with Cy3 dye.

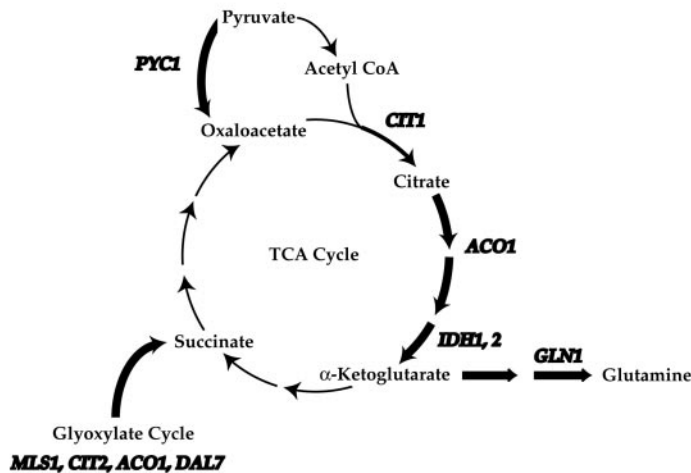
through anaplerotic reactions to maintain the respiratory competency of the cell (Stryer, 1995). These reactions include production of succinate via the glyoxylate cycle and formation of oxaloacetate directly from pyruvate, a reaction catalyzed by pyruvate carboxylase, encoded by the *PYCI* gene in yeast (Stucka et al., 1991; Walker et al., 1991). Remarkably, it is precisely the genes encoding these enzymes, as well as several TCA cycle enzymes that catalyze steps leading to the formation of  $\alpha$ -ketoglutarate, namely *ACO1*, *IDH1*, and *IDH2*, that we found to be repressed by glutamine (or glutamate) (Figs. 1 B and 3 A, and data not shown). Thus, one physiological response of cells growing in the absence of these nitrogen sources was increased expression of genes involved in these anaplerotic reactions. We decided to explore this regulation in greater detail.

Two distinct transcriptional regulatory complexes, namely *HAP* and *RTG*, have been demonstrated to regulate expression of *ACO1*, *IDH1*, and *IDH2* (Forsburg and Guarente, 1989; McNabb et al., 1995; Liu and Butow, 1999). We reasoned that the *RTG* genes were most likely

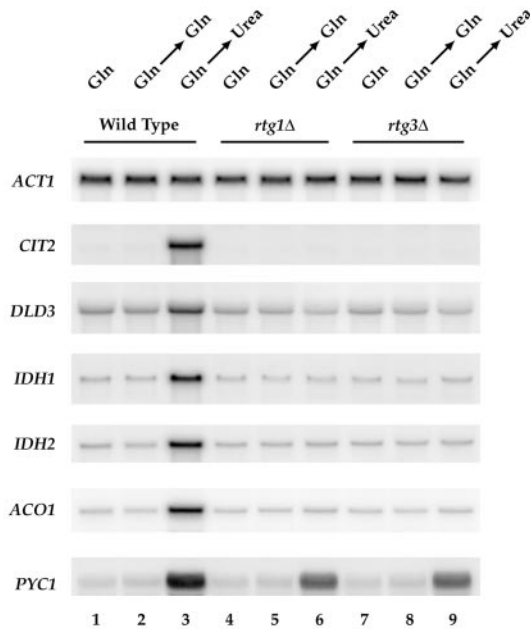
to be involved in nitrogen-regulated expression of these genes for the following reasons: (a) *HAP* gene control is subject to repression by glucose, which was used as a carbon source in all our experiments; (b) the *RTG* genes are responsible for regulating several other glutamine-repressed genes identified in our microarray experiments, including *CIT2* and *DLD3*; (c) *rtg* mutants are reported to be glutamate auxotrophs, a phenotype that would be consistent with our observations (Liu and Butow, 1999). The *RTG* family consists of three genes, *RTG1-3*, where *RTG1* and *RTG3* both encode members of the bZip family of transcription factors that form a heterodimeric complex (Jia et al., 1997; Rothermel et al., 1997). In agreement with results from a previous study (Liu and Butow, 1999), strains containing single deletions in either *RTG1* or *RTG3* grew very poorly on plates containing urea or ammonia as sole nitrogen sources, yet grew normally on plates containing glutamine or glutamate (data not shown).

To determine directly whether the *RTG* transcription factors were required for regulated expression of the

**A**



**B**

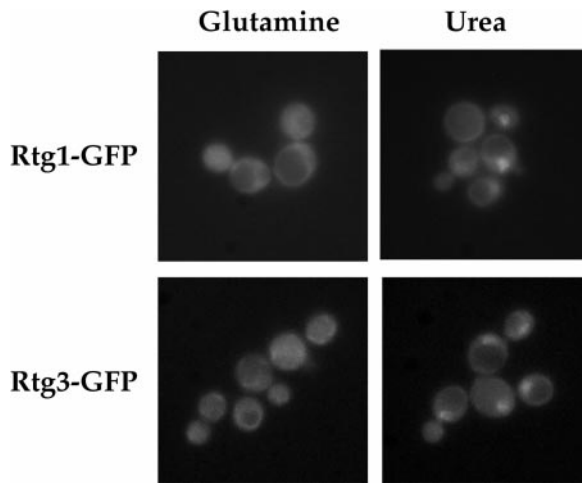


*Figure 3.* Rtg1 and Rtg3 are required for expression of distinct metabolic genes in MD-urea. (A) Summary of metabolic genes (bold) subject to glutamine-mediated transcriptional repression (see Table II; note that CIT1 is not listed in Table II as its MD-glutamine/MD-urea expression ratio of ~2.0 fell below the cut off value of 3.0 required for listing). Genes depicted were similarly repressed in MD-glutamine and MD-glutamate, except for GLN1 (see Fig. 1). (B) Nitrogen source shift experiment. Wild-type (S288c), *rtg1Δ* (PLY037), and *rtg3Δ* (PLY039) cells were grown in MD-glutamine until 0.5 OD<sub>600</sub>/ml and were either harvested (lanes 1, 4, and 7) or transferred to MD-glutamine (lanes 2, 5, and 8) or MD-urea (lanes 3, 6, and 9) media for 30 min before harvesting. RNA was prepared and analyzed by Northern blotting and probed for the specified mRNAs.

above metabolic genes under our experimental conditions, we performed the following nutrient-shift experiment. Wild type, *rtg1Δ*, or *rtg3Δ* cells were grown in MD-glutamine to early log phase, and were then transferred either to fresh MD-glutamine (as a control) or to MD-urea. Total RNA was isolated and mRNA levels of a representative number of these genes were analyzed by Northern blotting and normalized to actin mRNA levels (Fig. 3 B). As expected, each gene examined displayed increased expression, relative to actin, when wild-type cells were transferred to MD-urea but not to MD-glutamine (Fig. 3 B, compare lanes 1 and 2 with 3). In contrast, no increased expression of *ACO1*, *IDH1*, *IDH2*, *CIT2*, or *DLD3* was observed upon transfer of either *rtg1Δ* or *rtg3Δ* cells to MD-urea (Fig. 3 B, lanes 6 and 9). Interestingly, *PYC1* ex-

pression was increased in each mutant strain in MD-urea by about half the extent observed in wild-type cells (Fig. 3 B, compare lane 3 with 6 and 9). In addition, similar levels of expression were observed for *MLS1* in both wild-type and each *rtg* mutant strains in MD-urea (data not shown). These latter results demonstrate that factors in addition to the *RTG* genes are likely to be involved in the regulated expression of these two metabolic genes under these conditions and is consistent with previous biochemical and molecular genetic analyses of *RTG*-dependent control of *PYC1* expression (Small et al., 1995; Menendez and Gancedo, 1998). Taken together, these results revealed a strong correlation between *RTG*-dependent expression for a number of metabolic genes and the ability of yeast to grow using urea as a nitrogen source.



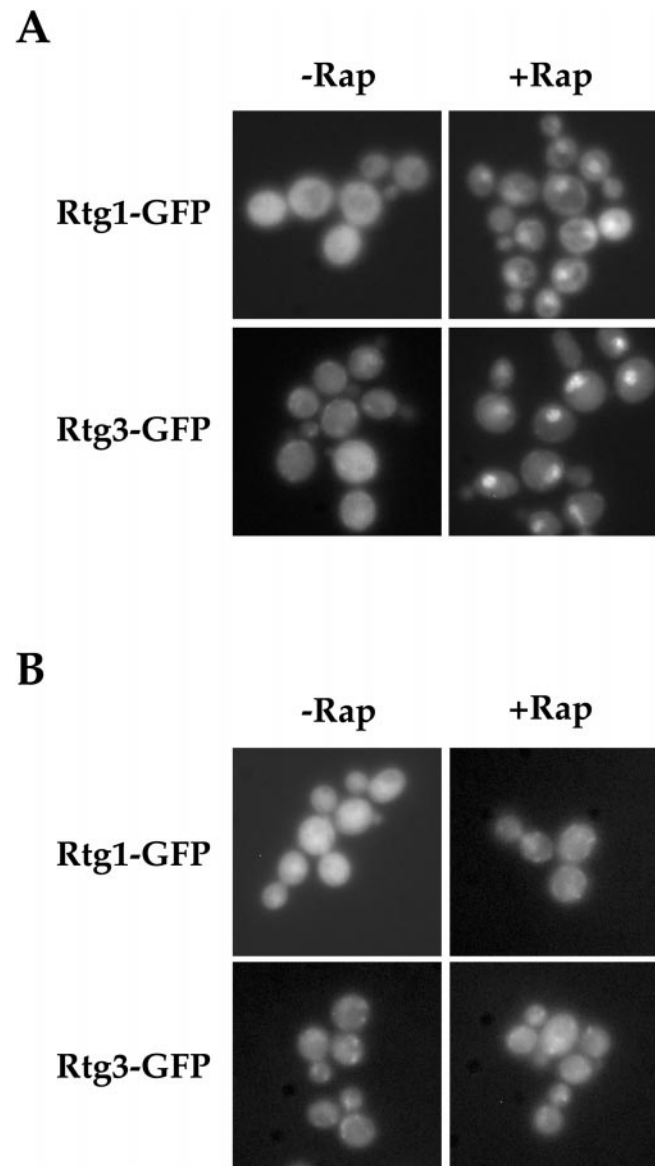


**Figure 4.** Rtg1 and Rtg3 are localized within the nucleus under glutamine-limiting conditions. *rtg1* $\Delta$  (EY0733) or *rtg3* $\Delta$  (EY0735) cells were transformed with pRtg1-GFP or pRtg3-GFP, respectively, and were grown to 0.5 OD<sub>600</sub>/ml in MD-glutamine or MD-urea and examined by fluorescence microscopy. Punctate nuclear fluorescence was observed for both Rtg1-GFP and Rtg3-GFP in MD-urea. The nuclear disposition of this GFP-based fluorescence was confirmed by its colocalization with DAPI-stained nuclear DNA (data not shown).

#### ***Nitrogen-dependent Changes in the Subcellular Localization of Rtg1 and Rtg3***

We wanted to understand the mechanism by which Rtg1 and Rtg3 activity is regulated. No significant differences were observed in the steady state levels of either *RTG1* or *RTG3* mRNAs, nor of Rtg1 or Rtg3 proteins in MD-glutamine versus MD-urea, suggesting that their activity was regulated post-translationally (data not shown; see below). Recently, a number of nutrient-responsive transcription factors have been shown to be regulated at the level of nuclear transport (reviewed in Beck and Hall, 1999; Kaffman and O'Shea, 1999). We therefore tested whether Rtg1 and/or Rtg3 might be similarly regulated in this manner. Toward this end, we fused the coding region of GFP in frame to the 3' ends of both *RTG1* and *RTG3* to produce fusion proteins, termed Rtg1-GFP and Rtg3-GFP. Fluorescence microscopy was then used to examine the subcellular localization of Rtg1-GFP and Rtg3-GFP in cells grown under different nitrogen conditions. Control experiments demonstrated that each fusion protein complemented the glutamine auxotrophy of its respective *rtg1* or *rtg3* deletion strain (data not shown).

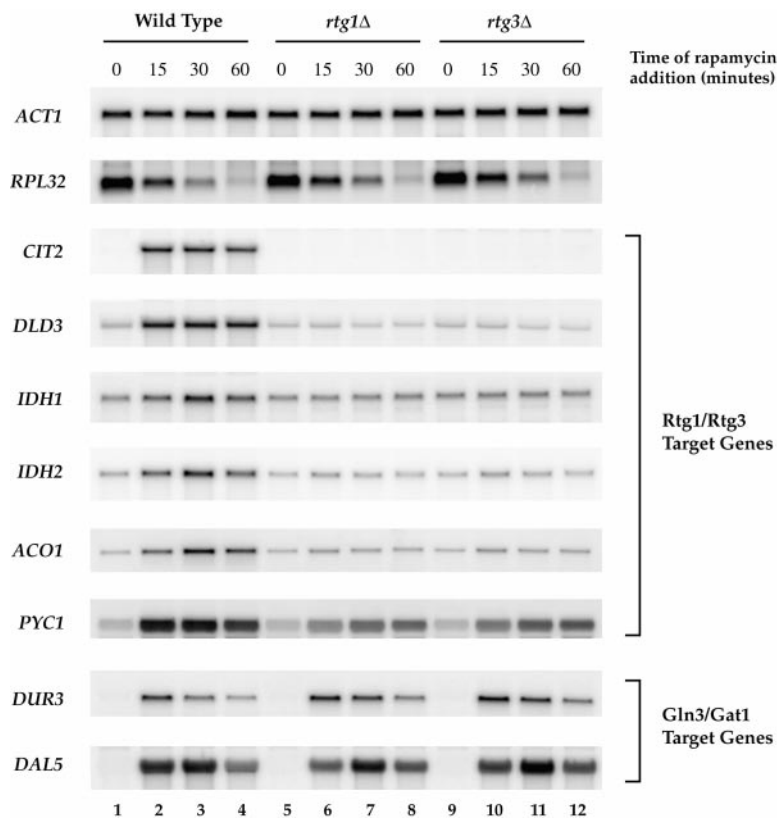
Both Rtg1-GFP and Rtg3-GFP appeared predominantly cytoplasmic when cells were grown in MD-glutamine (Fig. 4, left). Similar results were obtained when glutamate was used instead as a nitrogen source (data not shown). In contrast, both proteins were concentrated in the nucleus when cells were grown in MD-urea (Fig. 4, right). Also, in close agreement with the transcriptional responses described above, both Rtg1-GFP and Rtg3-GFP remained cytoplasmic when cells were grown in media that contained both glutamine and urea (data not shown). From these results, we conclude that *RTG*-dependent gene activation involves changes in the subcellular distribution of the Rtg1/Rtg3 complex.



**Figure 5.** Rtg1 and Rtg3 are localized within the nucleus after rapamycin treatment in a *TOR1*-dependent manner. (A) *rtg1* $\Delta$  (EY0733) or *rtg3* $\Delta$  (EY0735) cells expressing Rtg1-GFP or Rtg3-GFP, respectively, were treated with drug vehicle alone (left) or with 1  $\mu$ g/ml of rapamycin (right) for 5 min, followed by examination by fluorescence microscopy. Pronounced nuclear accumulation of both Rtg1-GFP and Rtg3-GFP was observed in cells treated with rapamycin. (B) The experiment in A was repeated using cells that carried the dominant rapamycin resistant *TOR1-1* allele.

#### ***Rtg1 and Rtg3 Activity and Subcellular Localization Is Regulated by the TOR Pathway***

We wished to identify the signaling pathway(s) that linked nitrogen quality to the localization of the Rtg1/Rtg3 complex. Here a clue was provided by the fact that many *RTG*-dependent target genes become induced when cells are treated with rapamycin, a specific inhibitor of the TOR kinases (Hardwick et al., 1999; C. Kao, T. Powers, P. Walter, G. Crabtree, and P. Brown, unpublished observations). Accordingly, we localized Rtg1-GFP and Rtg3-GFP in cells



**Figure 6.** Induction of *RTG*-dependent target genes by rapamycin. Wild-type (S288c), *rtg1Δ* (PLY037), and *rtg3Δ* (PLY039) cells were grown in MD-glutamine to 0.5 OD<sub>600</sub>/ml and were treated either with drug vehicle (lanes 1, 5, and 9) or with rapamycin for 15 (lanes 2, 6, and 10), 30 (lanes 3, 6, and 9), or 60 (lanes 4, 8, and 12) min. Cells were then harvested and RNA was prepared and analyzed by Northern blotting, probing for the specified mRNAs.

grown in nitrogen-rich media in both the absence and presence of rapamycin. As expected, both proteins remained in the cytoplasm in the absence of rapamycin (Fig. 5 A, left). In striking contrast, strong nuclear accumulation of both Rtg1-GFP and Rtg3-GFP was observed within 5 min of rapamycin addition (Fig. 5 A, right). Nuclear accumulation of these proteins by rapamycin was due specifically to inhibition of the TOR pathway, since little or no accumulation was observed when this experiment was repeated using a strain that contained the dominant rapamycin-resistant *TOR1-I* allele (Fig. 5 B, right). These results demonstrate that nuclear accumulation of Rtg1 and Rtg3 in rich nitrogen media is prevented by a functional TOR pathway.

A prediction of the above results was that inhibiting the TOR pathway might be sufficient to result in *RTG*-dependent gene activation. To test this directly, we performed a time course of rapamycin treatment of wild-type, *rtg1Δ*, and *rtg3Δ* cells grown in MD-glutamine, and then analyzed mRNA levels of several *RTG*-dependent targets by Northern blotting. In wild-type cells, each target gene examined showed increased expression within 15 min after addition of rapamycin (Fig. 6, compare lanes 1 and 2). The expression levels of these genes peaked at ~30 min and were comparable with the levels observed in cells grown in MD-urea (Fig. 6, lane 3; compare with Fig. 3 B, lane 3).

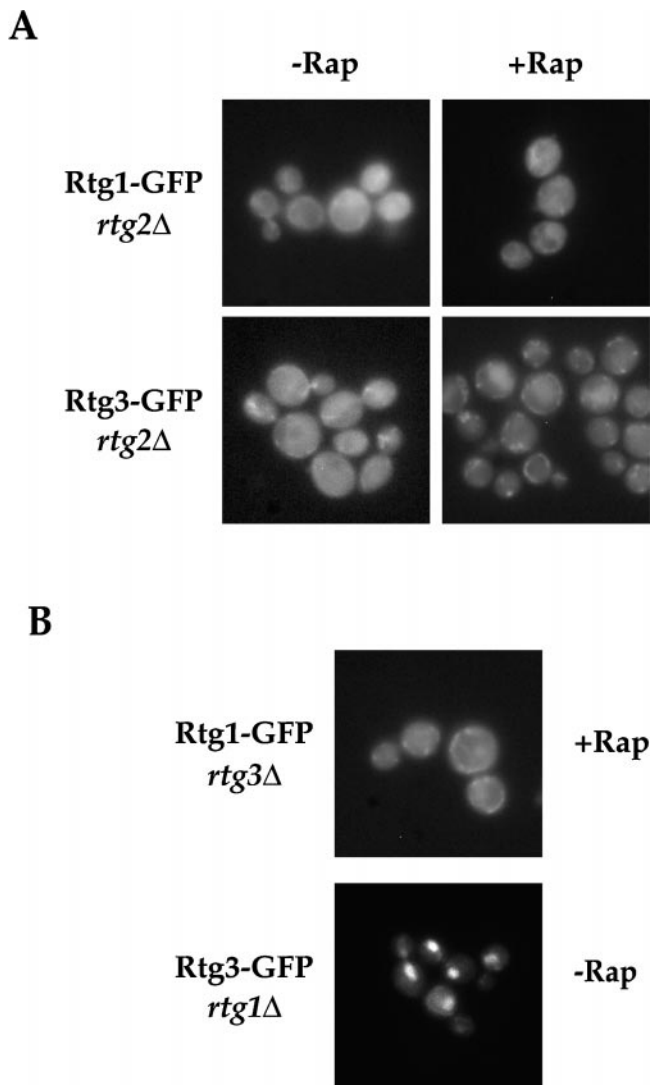
In striking contrast, no induction was observed for any of these genes when rapamycin was added to *rtg1Δ* and *rtg3Δ* cells (Fig. 6 B, lanes 5–12). The sole exception was *PYC1*, which showed a level of induction in each mutant strain of about half that observed in wild-type cells. This latter result is thus reminiscent of the behavior of *PYC1* in the nutrient shift experiment described above (Fig. 4 B) and indicates that an additional rapamycin-sensitive regu-

latory factor(s) is involved in the expression of this gene. As a control, we observed similar induction of two Gln3-dependent targets, *DUR3* and *DAL5*, in both wild-type and the *rtg* deletion strains, demonstrating that the loss of induction in the *rtg* mutants is specific for *RTG*-dependent targets. The specificity of these results was also confirmed by an observed decrease in *RPL32* mRNA levels in all strains after rapamycin treatment, as reported previously (Powers and Walter, 1999). Taken together, these results directly link TOR activity to both the subcellular localization and the activity of the Rtg1/Rtg3 complex.

Interestingly, deletion of the genes encoding the GATA transcriptional regulators Gln3 and Gat1, whose nucleocytoplasmic transport is similarly regulated by TOR, confers weak resistance to rapamycin (Beck and Hall, 1999). By contrast, we observed no change in the sensitivity of *rtg1Δ* or *rtg3Δ* cells to this drug, in comparison to wild-type cells (data not shown). Thus, we conclude that rapamycin-induced expression of *RTG*-dependent target genes does not contribute to the toxic effects of this drug on yeast cells.

### **Regulated Nucleocytoplasmic Transport of Rtg1 and Rtg3 Requires All Three RTG Genes**

The third member of the *RTG* gene family, *RTG2*, encodes a cytoplasmic protein that contains an HSP70-like ATP binding domain and displays homology to certain bacterial polyphosphatases and phosphatases (Liao and Butow, 1993; Koonin, 1994). Genetic evidence indicates that this gene functions upstream of *RTG1* and *RTG3* and is essential for expression of *RTG*-dependent target genes (Rothermel et al., 1997). We asked whether Rtg2 was involved in TOR-regulated nuclear accumulation of Rtg1



**Figure 7.** Regulated nucleocytoplasmic transport of Rtg1 and Rtg3 requires Rtg1, Rtg2, and Rtg3. (A) Rtg1-GFP and Rtg3-GFP were visualized in *rtg2Δ* (EY0734) cells in the absence (left) or presence (right) of rapamycin. In contrast to wild-type cells, neither Rtg1-GFP nor Rtg3-GFP relocate to the nucleus after rapamycin treatment (compare with Fig. 5 A). (B) Rtg1-GFP was visualized in *rtg3Δ* (EY0735) cells (top) and Rtg3-GFP was visualized in *rtg1Δ* (EY0733) cells (bottom). Note that Rtg1-GFP cannot accumulate in the nucleus upon rapamycin treatment in *rtg3Δ* cells. In contrast, Rtg3-GFP is localized constitutively to the nucleus in *rtg1Δ* cells, even in the absence of rapamycin addition.

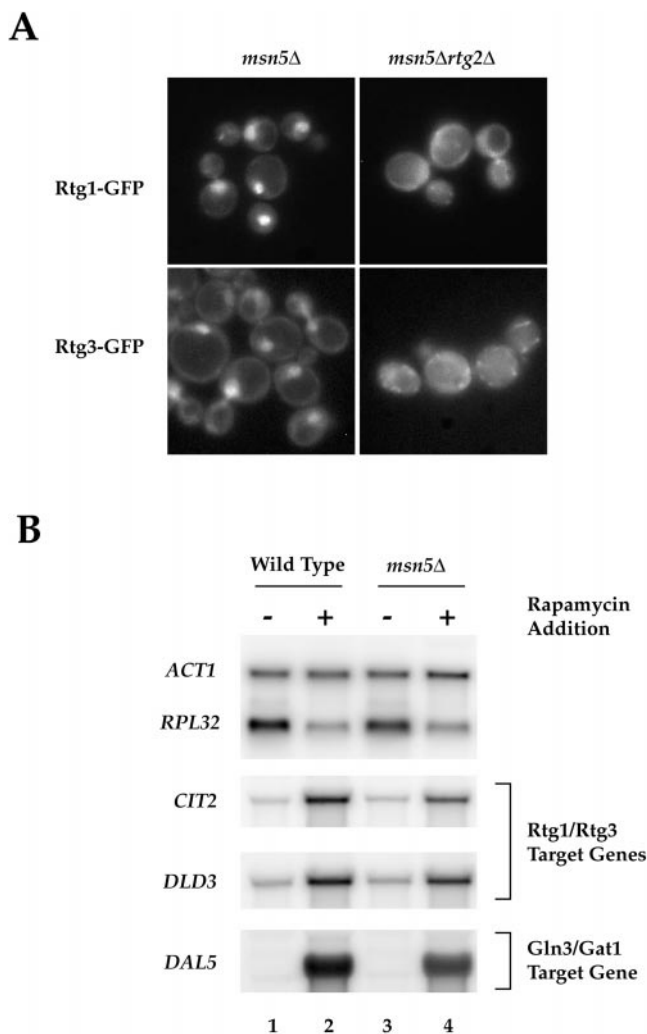
and Rtg3 by monitoring the localization of Rtg1-GFP and Rtg3-GFP in *rtg2Δ* cells, in both the absence and presence of rapamycin. As in wild-type cells, both proteins were localized to the cytoplasm in the absence of drug (Fig. 7 A, left). In contrast, no nuclear accumulation of either protein was observed after addition of rapamycin (Fig. 7 A, right). Additional experiments demonstrated that Rtg2 was itself a cytoplasmic protein and that its localization did not change after rapamycin treatment (data not shown). Thus, these results demonstrate that Rtg2 is essential for rapamycin-induced nuclear accumulation of the Rtg1/Rtg3 complex and that it carries out this function in the cytoplasm.

We next determined whether both Rtg1 and Rtg3 were required for TOR-regulated nuclear transport of the Rtg1/Rtg3 complex by monitoring the localization of Rtg1-GFP in *rtg3Δ* cells or, alternatively, Rtg3-GFP in *rtg1Δ* cells, both before and after rapamycin treatment. We observed that Rtg1-GFP remained exclusively cytoplasmic in *rtg3Δ* cells, even after rapamycin addition (Fig. 7 B, top, and data not shown). Since Rtg1 is a relatively small protein of 177 amino acids, we wanted to exclude the possibility that the constitutive presence of Rtg1-GFP in the cytoplasm in *rtg3Δ* cells was not due simply to the diffusion of this protein out of the nucleus after rapamycin treatment. Toward this end, we fused three tandem copies of the coding region of GFP to the 3' end of the *RTG1* gene to create a much larger protein, termed Rtg1-GFP<sub>3</sub>. Control experiments confirmed that this fusion protein was functional (data not shown). We observed that Rtg1-GFP<sub>3</sub> also remained in the cytoplasm in rapamycin-treated cells, indicating that Rtg1 cannot accumulate in the nucleus in the absence of Rtg3 (data not shown).

In striking contrast, we observed that Rtg3-GFP was localized exclusively in the nucleus in *rtg1Δ* cells, in both the presence and absence of rapamycin (Fig. 7 B, bottom, and data not shown). Thus, regulated transport of the Rtg1/Rtg3 complex requires that both proteins be present together. One potential explanation to account for the constitutive localization of these two proteins in different subcellular compartments is that Rtg1 contains a nuclear export signal, whereas Rtg3 contains the nuclear import signal (NLS) for the heterodimer. Consistent with this interpretation, a recent study has confirmed that the basic domain of the bHLH motif of Rtg3 contains a functional NLS (Sekito et al., 2000). Interestingly, however, we observed that Rtg3 was also localized constitutively to the nucleus when cells were deleted for both *RTG1* and *RTG2* (data not shown). Given that Rtg3 is localized exclusively in the cytoplasm when cells are singly deleted for *RTG2* (Fig. 7 A), together these results suggest that Rtg1 may play a more antagonistic role in regulating Rtg3 nuclear import. A similar conclusion has also been reached recently by Sekito et al. (2000).

#### **Export of Rtg1 and Rtg3 from the Nucleus Requires the $\beta$ -Importin Homologue Msn5**

Constitutive localization of Rtg3 in the nucleus in *rtg1Δ* cells suggested that export of the Rtg1/Rtg3 complex from the nucleus might play a role in the regulation of these transcription factors. Previous studies of another nutrient-responsive transcription factor, Pho4, has demonstrated that its export from the nucleus depends on the activity of Msn5, a member of the  $\beta$ -importin family of nuclear receptors (Kaffman et al., 1998a). We therefore tested whether export of Rtg1 and Rtg3 might similarly be regulated by this protein. Indeed, we observed that both Rtg1-GFP and Rtg3-GFP were constitutively nuclear in *msn5Δ* cells, demonstrating that this factor is required, either directly or indirectly, for export of the Rtg1/Rtg3 complex from the nucleus (Fig. 8 A, left). Interestingly, when we examined the localization of Rtg1-GFP and Rtg3-GFP in *msn5Δ rtg2Δ* cells, both proteins remained in the cytoplasm, consistent with the above observation that Rtg2 is



**Figure 8.** (A) Msn5 is required for export of Rtg1 and Rtg3 from the nucleus. (A) *msn5Δ* (EY0736) and *msn5Δ rtg2Δ* (EY0744) cells were transformed with pRtg1-GFP (top) or pRtg3-GFP (bottom) and grown to 0.5 OD<sub>600</sub>/ml in SCD media lacking uracil and were examined by fluorescence microscopy. Rtg1-GFP and Rtg3-GFP were localized constitutively within the nucleus in *msn5Δ* cells but not in *msn5Δ rtg2Δ* cells. (B) *RTG*-dependent target genes remain responsive to TOR signaling in *msn5Δ* cells. Wild-type (K699) and *msn5Δ* (EY0736) cells were grown in YPD until 0.5 OD<sub>600</sub>/ml and were treated either with drug vehicle (lanes 1 and 3) or with rapamycin (lanes 2 and 4) for 30 min (this time of incubation corresponded to the peak induction of *RTG*-dependent gene expression observed in the rapamycin time course in Fig. 6). Cells were then harvested and RNA was prepared and analyzed by Northern blotting, probing for the specified mRNAs.

absolutely required for nuclear entry of the Rtg1/Rtg3 complex (Fig. 8 A, right). Additional control experiments demonstrated that Rtg2-GFP remained in the cytoplasm in *msn5Δ* cells (data not shown).

If regulated access to the nucleus represents the primary mechanism by which the activity of the Rtg1/Rtg3 complex is controlled, then we expected to observe constitutive activation of their target genes in *msn5Δ* cells in the absence of rapamycin. However, no such increased expression of *RTG*-target genes was observed in *msn5Δ* cells

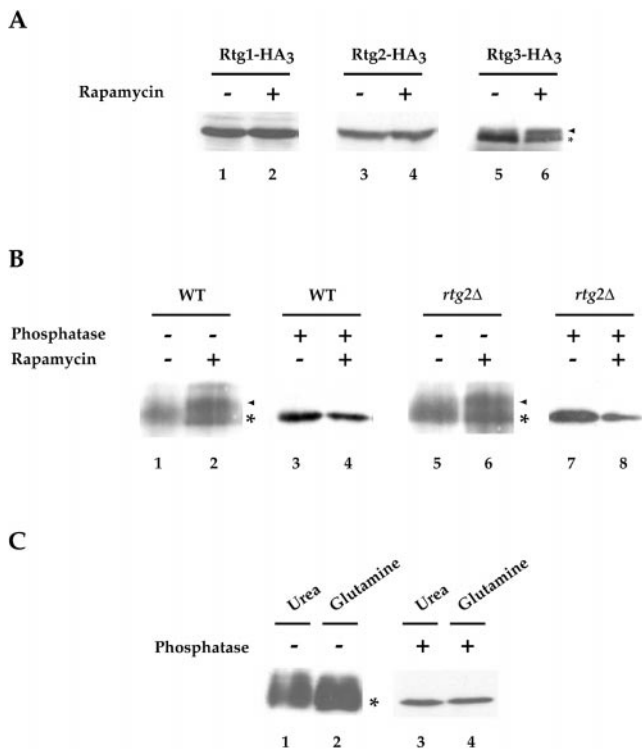
compared with wild type (Fig. 8 B, compare lanes 1 and 3). This observation is consistent with studies of other regulated transcription factors; namely, that constitutive nuclear localization does not necessarily result in gene activation and that other regulatory mechanisms are involved (Komeili and O'Shea, 1999). Remarkably, however, we observed rapid induction of *RTG*-dependent target genes in *msn5Δ* cells after addition of rapamycin (Fig. 8 B, compare lanes 3 and 4). This latter result demonstrates that despite its steady state nuclear localization in *msn5Δ* cells, the Rtg1/Rtg3 complex remains responsive to changes in TOR signaling.

#### *TOR-dependent Changes in the Phosphorylation State of Rtg3*

Previous studies have demonstrated that changes in phosphorylation of a transcription factor can be important for regulating its activity as well as concentration in the nucleus (Beck and Hall, 1999; Komeili and O'Shea, 1999). We therefore wished to determine whether rapamycin-induced nuclear accumulation of Rtg1 and Rtg3 also correlated with changes in the phosphorylation state of either of these proteins. Toward this end, we used immunoblot analysis to monitor the electrophoretic mobility of these proteins, as well as Rtg2, from extracts in which each protein was genomically tagged at its COOH terminus with the HA<sub>3</sub> epitope (see Materials and Methods). Extracts were prepared from cells grown in rich nitrogen media that had been incubated with drug vehicle alone or with rapamycin, and then subsequently prepared in either the presence or absence of phosphatase inhibitors. No change in the relative abundance or mobility of Rtg1-HA<sub>3</sub> or Rtg2-HA<sub>3</sub> was observed in untreated versus rapamycin-treated cells (Fig. 9 A, lanes 1–4). In contrast, a slower migrating form of Rtg3-HA<sub>3</sub> appeared after addition of rapamycin, which was observed only when extracts were prepared in the presence of phosphatase inhibitors (Fig. 9 A, compare lanes 5 and 6, data not shown). These results suggested that inhibition of TOR signaling resulted in a change in the phosphorylation state of Rtg3.

We wanted to confirm that Rtg3 was a phosphoprotein by treating purified Rtg3 with phosphatases *in vitro*; however, the HA epitope-tagged form of this protein was not efficiently immunoprecipitated from cell extracts. We therefore used a form of Rtg3 that was fused at its COOH terminus to two z domains from Protein A, termed Rtg3-zz, which could be immunoprecipitated quantitatively from extracts using immobilized-IgG (data not shown). The results confirmed that rapamycin treatment resulted in the conversion of a portion of Rtg3-zz to a more slowly migrating form (Fig. 9 B, compare lanes 1 and 2). Moreover, this slower form was abolished after treatment with phosphatase, confirming that the rapamycin-induced change in electrophoretic mobility of Rtg3-zz is due to changes in phosphorylation (Fig. 9 B, compare lanes 2 and 4; arrowhead, slower migrating form). Interestingly, the rapamycin-induced shift in Rtg3 mobility was also observed in *rtg2Δ* cells, suggesting that TOR influences Rtg3 phosphorylation independently from Rtg2 function (Fig. 9 B, compare lanes 5 and 6).

While the above results demonstrate that TOR influences the phosphorylation state of Rtg3, several other re-



**Figure 9.** Rtg3 is a phosphoprotein and is differentially phosphorylated after rapamycin treatment. (A) Cells expressing Rtg1-HA<sub>3</sub> (PLY047), Rtg2-HA<sub>3</sub> (PLY089), and Rtg3-HA<sub>3</sub> (PLY050) were grown to 0.5 OD<sub>600</sub>/ml in YPD and were treated either with drug vehicle alone or with rapamycin for 15 min. Extracts were prepared and Western blot analysis was performed using anti-HA monoclonal antibodies to detect each protein. No change in the abundance or relative mobility of Rtg1 or Rtg2 could be detected after rapamycin treatment. In contrast, a portion of Rtg3 showed an increased mobility (arrowhead) after rapamycin treatment, compared with its mobility in the absence of rapamycin (\*). (B) Wild-type (K699) and *rtg2Δ* (EY0734) cells transformed with pRtg3-zz and were grown to 0.5 OD<sub>600</sub>/ml in SCD media lacking uracil. Cells were then treated with drug vehicle or with rapamycin for 15 min. Extracts were prepared and Rtg3-zz was immunoprecipitated with IgG-Sepharose and either mock-treated or treated with phosphatase before Western blot analysis, as indicated. Increased mobility of a portion of Rtg3-zz after rapamycin treatment is indicated (arrowhead). (C) Wild-type (K699) cells carrying pRtg3-zz were grown to 0.5 OD<sub>600</sub>/ml in MD-glutamine or MD-urea and processed as in B. For each experiment in A–C, all samples were from the same gel. Identical results were obtained in three separate experiments.

sults indicate that phosphorylation-dependent regulation of this protein is likely to be complex. First, we observed that Rtg3-zz was phosphorylated in the absence of rapamycin treatment, a conclusion based on its increased mobility after phosphatase treatment, suggesting that Rtg3 is likely to be phosphorylated on multiple sites (Fig. 9 B, compare lanes 1 and 3). The existence of multiple sites of phosphorylation could account for the relatively poor resolution of Rtg3 on SDS-PAGE gels in the absence of phosphatase treatment. Second, we were unable to detect a difference in the mobility of Rtg3-zz in cells grown in MD-glutamine versus MD-urea (Fig. 9 C, lanes 1 and 2). This latter result suggests either that rapamycin-induced

changes are transient or that additional changes in the phosphorylation state of this protein occur during steady state growth of cells in media lacking glutamine. Finally, multiple levels of regulation, possibly mediated by distinct changes in the phosphorylation state of Rtg3, would be consistent with our above observation that the Rtg1/Rtg3 complex is concentrated in the nucleus in *msn5Δ* cells, yet their target genes remain uninduced. Identifying the residue(s) of Rtg3 that are phosphorylated under these different conditions will be required to resolve these issues.

## Discussion

Genome-wide expression analysis represents a powerful approach for exploring the scope of transcriptional regulation associated with different biological processes. The appeal of this method is most often attributed to the enormous amount of information that it can yield, as exemplified by studies in yeast of the diauxic shift, sporulation, and the cell cycle, where each of these processes involves sequential changes in the expression of groups of coregulated genes that number well into the hundreds (DeRisi et al., 1997; Chu et al., 1998; Spellman et al., 1998). By contrast, our results presented here demonstrate that by studying the effects of subtle changes in growth conditions (e.g., by varying the source of assimilable nitrogen), microarray analysis can help bring a specific biological problem into sharper focus by identifying a concise set of coregulated genes.

Specifically, we have demonstrated that a surprisingly small number of genes are differentially expressed when yeast cells use glutamine versus urea as their sole source of nitrogen (Table II). All of these genes are either induced or repressed by the presence of glutamine, a fact that is consistent with its established importance as a major regulator of nitrogen metabolism (Magasanik, 1992; Marzluf, 1997). One prominent group of glutamine-repressed genes encodes proteins involved in the uptake and metabolism of alternative sources of nitrogen, including urea and allantoin, and whose expression is controlled by the Gln3 and Ure2 regulatory proteins (Coffman et al., 1997; Cox et al., 1999). Recent studies have demonstrated that these genes are negatively regulated by the TOR signaling pathway via a mechanism that involves sequestration of the transcription factor Gln3 in the cytoplasm (Beck and Hall, 1999).

A second prominent group of glutamine-repressed genes encodes enzymes involved in the TCA and glyoxylate cycles and whose expression in urea-containing media requires the heterodimeric transcription factors Rtg1 and Rtg3. Differential expression of these metabolic genes is consistent with the proposal by Liu and Butow (1999) that one important role of RTG-dependent gene expression is to provide adequate levels of  $\alpha$ -ketoglutarate for use in the de novo biosynthesis of glutamate, which in turn is required for the synthesis of glutamine. In addition, our results extend involvement of RTG-dependent regulation to include *PYCI*, which provides an alternative route for the synthesis of oxaloacetate for use in both the TCA cycle and in amino acid biosynthesis (Fig. 3). These results thus highlight the intimate relationship that exists between nitrogen and carbon metabolism as well as its importance to normal cell growth.

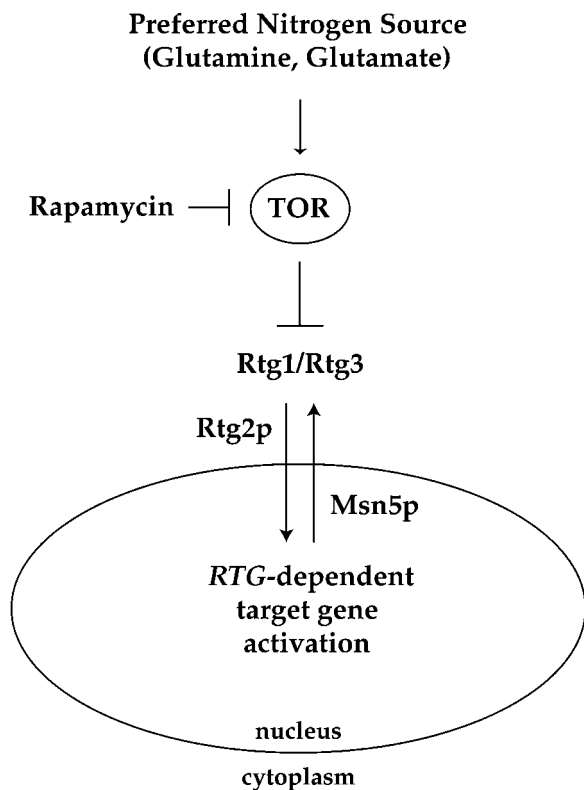


Figure 10. Model for involvement of the TOR pathway in nitrogen-dependent regulation of the Rtg1 and Rtg3 transcription factors. See text for details.

Remarkably, we find that TOR signaling also regulates *RTG*-dependent gene activity. Specifically, inhibition of the TOR kinases by rapamycin results in both rapid nuclear accumulation of the Rtg1/Rtg3 complex as well as induction of their target genes. In addition, rapamycin treatment correlates with changes in the phosphorylation state of Rtg3, indicating that nucleocytoplasmic transport of the complex is likely to be regulated by differential phosphorylation of Rtg3. These results thus contribute to a growing body of evidence demonstrating that one important role of TOR is to control the activity of specific nutrient-responsive transcription factors. Moreover, our findings suggest that TOR signaling may provide an important mechanism by which carbon and nitrogen use are linked. Whether Rtg3 phosphorylation is influenced by the activity of the Tap42-Sit4 phosphatase complex, as has been shown recently for Gln3 (Beck and Hall, 1999), remains to be determined.

Our results also shed light on the functional role of Rtg2 in regulating *RTG*-dependent gene expression, a previously identified upstream positive regulator of Rtg1 and Rtg3 (Liao and Butow, 1993; Rothermel et al., 1997). Specifically, we find that Rtg2 is required for rapamycin-induced entry of the Rtg1/Rtg3 complex into the nucleus (Fig. 10). How Rtg2 acts mechanistically is unclear at present; however, we believe that Rtg2 is likely to be required for a step subsequent to the action of TOR, based upon the observation that rapamycin-induced changes in Rtg3 phosphorylation are observed in *rtg2Δ* mutant cells (Fig. 8 B). Furthermore, our examination of Rtg3 localization in *rtg1Δ*, *rtg2Δ*,

and *rtg1Δ rtg2Δ* mutant cells suggests that Rtg2 may interact primarily with Rtg1 to regulate nuclear entry of the Rtg1/Rtg3 complex (Fig. 7, and data not shown).

In contrast to the role played by Rtg2, we find that Msn5, a member of the importin- $\beta$  family of nuclear transport receptors, is required for nuclear export of the Rtg1/Rtg3 complex (Fig. 10). Thus, in *msn5Δ* cells, both Rtg1 and Rtg3 are constitutively localized to the nucleus. We do not know yet whether Msn5 interacts directly with Rtg1 and/or Rtg3 to facilitate their export from the nucleus, as has been demonstrated for Pho4 (Kaffman et al., 1998). It is possible that Msn5 is instead required for the proper localization of another protein(s) that is involved directly in the export of the Rtg1/Rtg3 complex.

While nucleocytoplasmic transport of the Rtg1/Rtg3 complex is essential for regulated *RTG*-dependent gene activation, our results indicate that additional control mechanisms are involved. This conclusion is based on our analyses of *msn5Δ* cells, where both Rtg1 and Rtg3 are concentrated in the nucleus yet their target genes remain uninduced (Fig. 9). As these genes can nevertheless be induced rapidly in *msn5Δ* cells following rapamycin treatment, these results raise the intriguing possibility that TOR regulates Rtg1/Rtg3 activity in both the cytoplasm as well as in the nucleus.

*RTG1-RTG3* were originally identified as genes required for increased expression of *CIT2* under conditions where mitochondrial respiratory function is impaired, as in  $\rho^0$  petite mutants that lack mitochondrial DNA (Liao et al., 1991; Liao and Butow, 1993). This process, termed retrograde regulation, is believed to involve intracellular signaling from the mitochondria to the nucleus (Liao and Butow, 1993). Subsequently, the *RTG* genes have been shown to be involved in the expression of several genes that encode peroxisomal proteins in addition to glyoxylate cycle enzymes (Chelstowska and Butow, 1995; Kos et al., 1995; McCammon, 1996; Liu and Butow, 1999). Indeed, the results of these studies suggest that both mitochondrial and peroxisomal functions are intimately linked via *RTG*-dependent gene expression. An important question prompted by our present results thus concerns the relationship between TOR signaling and retrograde regulation. While this manuscript was in preparation, Sekito et al. (2000) reported that Rtg1 and Rtg3 are localized constitutively to the nucleus in  $\rho^0$  cells. Moreover, this localization depends on a functional Rtg2 protein. Given these similarities to what we present here, one possibility is that TOR may be integrated with retrograde signaling.

At odds with this conclusion, however, is the finding that nuclear accumulation of the Rtg1/Rtg3 complex in  $\rho^0$  cells appears to correlate with a substantial decrease in phosphorylation of Rtg3 (Sekito et al., 2000). By contrast, our results suggest that nuclear import of this complex after rapamycin treatment may coincide with increased phosphorylation of Rtg3. Moreover, and very importantly, we were unable to detect a difference in the mobility of Rtg3 on SDS-PAGE following the growth of cells in glutamine- versus urea-containing media (Fig. 9 C). Nevertheless, the Rtg1/Rtg3 complex is localized in different subcellular compartments, cytoplasm versus nucleus, under these different nitrogen conditions (Fig. 4). Thus, the precise relationship between the phosphoryla-

tion state of Rtg3 and its nucleocytoplasmic disposition needs further clarification, which will require identification of residues in Rtg3 that are subject to phosphorylation under different cellular conditions. At present, it thus remains possible that retrograde and TOR signaling may represent independent routes by which Rtg1 and/or Rtg3 are regulated and that both pathways ultimately converge on the same nucleocytoplasmic transport step.

Inhibition of the TOR kinases by rapamycin affects the expression of a large number of genes, including those regulated by nitrogen and glucose-sensitive signaling pathways, glycolytic genes, genes expressed during the diauxic shift, and r-protein and rRNA genes (Beck and Hall, 1999; Cardenas et al., 1999; Hardwick et al., 1999; Powers and Walter, 1999; C. Kao, T. Powers, P. Walter, G. Crabtree, P. Brown, unpublished observations). By contrast, we find that only a small subset of these targets are expressed differentially in our nitrogen source experiments, in particular the *GLN3*- and *RTG*-dependent genes described above. These observations suggest that distinct branches of the TOR pathway may be regulated independently according to the precise nutritional state of the cell and, moreover, suggest that TOR activity may be modulated by multiple upstream nutritional signals. On the other hand, many of the glutamine-regulated genes identified in this study are affected to some extent by rapamycin treatment (T. Powers, unpublished results). Thus, we believe that TOR signaling is likely to represent an important mechanism by which the availability of glutamine and/or glutamate is coupled to changes in gene expression in eukaryotic cells. One challenge now at hand is to understand how TOR activity is regulated under different nitrogen conditions. Such studies will undoubtedly prove invaluable toward deciphering the role that this pathway plays in promoting normal cell growth.

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