Gln3p is a GATA-type transcription factor responsive to different nitrogen nutrients and starvation in yeast *Saccharomyces cerevisiae*. Recent evidence has linked TOR signaling to Gln3p. Rapamycin causes dephosphorylation and nuclear translocation of Gln3p, thereby activating nitrogen catabolite repressible-sensitive genes. However, a detailed mechanistic understanding of this process is lacking. In this study, we show that Tor1p physically interacts with Gln3p. An intact TOR kinase domain is essential for the phosphorylation of Gln3p, inhibition of Gln3p nuclear entry and repression of Gln3p-dependent transcription. In contrast, at least two distinct protein phosphatases, Pph3p and the Tap42p-dependent phosphatases, are involved in the activation of Gln3p. The yeast pro-prion protein Ure2p binds to both hyper- and hypo-phosphorylated Gln3p. In contrast to the free Gln3p, the Ure2p-bound Gln3p is significantly resistant to dephosphorylation. Taken together, these results reveal a tripartite regulatory mechanism by which the phosphorylation of Gln3p is regulated.

Yeast cells have sophisticated transcriptional programs to regulate the expression of genes encoding proteins responsible for the transport and degradation of small nitrogenous compounds in response to different nitrogen nutrients. Glutamine is a preferred nitrogen source, whereas glutamate is a poor nitrogen source. Cells respond to the preferred nitrogen sources by decreasing the expression of genes associated with transport and degradation of poor nitrogen sources. For example, glutamine prevents the expression of *GLN1* and *GAP1*, structural genes for glutamine synthetase and the general amino acid permease. In contrast, their expression is derepressed with glutamate as the sole nitrogen source. This physiological control is designated nitrogen catabolite repression (NCR)\(^1\) (reviewed in Ref. 1). Gln3p is a GATA-type transcriptional factor responsible for the expression of a majority of NCR-sensitive genes. Gln3p activity is inhibited by Ure2p, a yeast pro-prion protein in the presence of preferred nitrogen sources (1, 2). Other transcriptional factors are also involved in the complex regulation of NCR-sensitive genes, including the four GATA-type transcriptional factors Gln3p, Gaf1p, Dal80p, and Gzf3p (3), and the Zinc finger transcriptional factors Dal81p and Dal82p (4). Each NCR-sensitive gene appears to be regulated in a combinatorial manner by several transcription factors.

Rapamycin is a macrocyclic antibiotic that has potent immunosuppressive effects. When complexed with its immunophilin receptor FKBP12, rapamycin causes growth inhibition of both mammalian cells and yeast. There are two highly related TOR proteins (targets of rapamycin), Tor1p and Tor2p in budding yeast (5–7). They are members of the ATM (*ataxia telangiectasia* mutated)-related family and possess intrinsic protein Ser/Thr kinase activity (8–10). FKBP12-rapamycin binds to the FKB12-rapamycin-binding domain of TOR proteins (11) and inhibits the protein kinase activity of Tor1p (8). The best characterized function of TOR is its role in translation initiation (12, 13). However, emerging evidence indicates that TOR signaling is rather complex and possibly involved in many other cellular processes. For example, several studies show that TOR signaling is clearly involved in regulation of genes involved in ribosomal biogenesis (14–16) and NCR (17–19). TOR signaling to NCR genes has been linked to the GATA-type transcription factor Gln3p and its inhibitor Ure2p (17–19). Rapamycin causes rapid dephosphorylation and nuclear entry of Gln3p (18). However, it is not clear what the role of TOR is with regard to the regulation of Gln3p. Although the Tap42p-Sit4p protein phosphatase complex has been suggested to mediate TOR signaling to Gln3p (18), there is also evidence indicating that it is not involved (19).

In this study, we show that TOR binds to Gln3p via its HEAT domain. An intact TOR kinase domain is required for phosphorylation of Gln3p, the inhibition of Gln3p nuclear translocation, and repression of Gln3p-dependent genes. We also show that mutations in at least two phosphatases, Tap42p-Sit4p and Pph3p, can affect the activation of Gln3p. We further demonstrate that Ure2p is capable of binding to both phosphorylated and dephosphorylated Gln3p but appears to protect Gln3p from dephosphorylation. Therefore, a tripartite regulatory mechanism is involved in governing the repression and activation of Gln3p.

### MATERIALS AND METHODS

**Two-hybrid Library Screens, Plasmids, and Strains**—To create a Gal4p DNA-binding domain-Tor1p fusion plasmid, pYDF72 (11) was modified inserting a NOT1 restriction site into the SacII site. The pAS2–2 was created by inserting a NOT1 linker into the EcoRI site of pAS2–1 (CLONTECH, Inc.). The full-length TOR1 was excised with NOT1 and PstI and inserted into the same sites of pAS2–2 to create pASTOR1. To identify Tor1p-interacting proteins, we performed a yeast two-hybrid screen with full-length Tor1p fused to the DNA-binding domain (BD) of Gal4p (BD-Tor1p). A plasmid library of fusions between the transcription activation domain (AD) of Gal4p and yeast genomic DNA fragments (20) was screened for interaction with BD-Tor1p in a yeast reporter strain. The positive clones specifically interacted with...
Regulation of Gln3p by TOR, Ure2p, and Phosphatases

BD-Tor1p but not with a BD alone, the BD-human lamin C, or p53 fusion proteins. Sequence analyses of the positive clones were carried out by automatic DNA sequencing. The DNA sequences were used to search for open reading frames in the Saccharomyces Genomic Data Base. A Myc epitope tag or protein A (ProA) tag was introduced at the C terminus of Gln3p and Ure2p, respectively, on the chromosomal copies of GLN3 and URE2 genes using a method developed by Schneider et al. (21). Deletion of GLN3 and URE2 were carried out by polymerase chain reaction as described previously (22).

**GST Fusion Proteins—URE2** was excised from PJ6, a library clone containing the URE2 insert with BamHI, and inserted into pGEX-4T1 to create pGEX-Ure2a2aa9-243, respectively. pGEX-Gln3(510–720aa) was similarly created by exciting the genomic inserts from pJ2, an Escherichia coli library clone containing the chromosomal GLN3 genomic DNA fragment. GST, GST-Ure2p, and GST-Gln3p were prepared according to the manufacturer's manual (Amersham Pharmacia Biotech). Immobilized GST fusion proteins on glutathione-Sepharose beads were used directly for assaying their abilities to bind to Tor1p-Myc.

**Northern Blotting Analysis—** Exponential wild type and mutant yeast cultures were treated with 200 nM rapamycin, FK506, or a drug vehicle control (methanol). Aliquots of yeast cultures were withdrawn at different times. Total yeast RNAs were prepared using the phenol freezing extraction method (23). 20 μg of total yeast RNA samples were separated on denaturing agarose gels, transferred onto nylon filters, hybridized to 32P-labeled DNA probes, and detected by phosphorimaging.

**Gene Expression Profiling—** Yeast strains carrying an integrated URA3 cassette were transformed with p415GAL1::GALACT and p415GAL1::GALACT::ura3-52 plasmids. Yeast cultures expressing the Gal4p fusion proteins were incubated with 200 nM rapamycin. Samples were withdrawn at different times, and total yeast RNA was isolated and used to prepare 32P-labeled cDNA probes by reverse transcription. The radioactively labeled probes were used to hybridize GeneFilters® (Research Genetics, Inc.) that contain a microarray of the entire yeast open reading frames. The results were analyzed by the QuantityOne software package (Bio-Rad).

**ImmunoPrecipitation, and Phosphatase Treatment—** Log phase yeast cells were harvested and lysed with glass beads in disruption buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 plus a mixture of protease inhibitors; Roche Molecular Biochemicals) by vortexing. 1 mg yeast extracts were used for immunoprecipitation with 5 μg of monoclonal antibody (mAb) 9E10 for Myc. For Western blotting analysis, 20 μg protein samples were used for gel electrophoresis and detected by ECL (Amersham Pharmacia Biotech). For the phosphatase treatment, the Gln3p-Myc immunocomplex was incubated with CIP buffer alone, 20 units of CIP (Roche Molecular Biochemicals), or 20 units CIP plus phosphatase inhibitor 10 μM Na2P2O7 for 10 min at 30 °C.

**Immunofluorescence—** Yeast immunofluorescence studies were performed as described without dehydration (24). Myc-specific mAb 9E10 were used at 1:500 dilution. A rabbit anti-mouse IgG conjugated to Alexa Fluor 488 was used for visualization. Images were captured by a SPOT digital camera.

**RESULTS**

To elucidate the mechanisms of TOR signaling, we initiated a yeast two-hybrid screen to identify Tor1p-interacting proteins. Gln3p and Ure2p were isolated multiple times as independent clones. They interacted with Tor1p and Tor2p but not with a variety of control proteins (Fig. 1a). We have also found that Tor2p interacted with Gln3p and Ure2p (Fig. 1a), suggesting that both Tor1p and Tor2p are involved in the Gln3p-dependent process. We further studied their interaction biochemically. Gln3p and Ure2p were produced as GST fusion proteins in Escherichia coli and incubated with lysates from yeast expressing Tor1p-Myc. We found that Tor1p-Myc bound to both GST-Gln3p and GST-Ure2p but not to GST alone (Fig. 1b). By deletional analysis, we localized the Gln3p- and Ure2p-binding sites to an N-terminal fragment of Tor1p (Fig. 1c) that contains multiple HEAT repeats previously predicted to mediate protein-protein interaction (25). We further found that the ∆gln3 mutation caused rapamycin resistance, whereas deletion of URE2, the inhibitor of GLN3 rendered hypersensitivity to rapamycin (Fig. 1d). These data suggest an inhibitory role of TOR in the regulation of Gln3p. Activation of Gln3p by rapamycin in rich medium (such as YPD) may cause starvation response and contribute to growth inhibition by rapamycin.

To further examine whether TOR signaling is involved in regulating Gln3p, we performed Northern blotting analysis of two NCR-sensitive genes GAP1 (the general amino acid permease) and GLN1 (glutamine synthetase) that are known to be Gln3p-dependent. In agreement with the previous studies (17–19), we found that their expression was rapidly induced by rapamycin (Fig. 2a). Their expression was significantly reduced in the ∆gln3 strain, but their expression became constitutive in the ure2 genetic background. Thus, the effect of rapamycin is Gln3p-dependent (Fig. 2b). However, we have consistently noted a small residual induction of GAP1 and GLN1 by rapamycin in the absence of Gln3p (Fig. 2b). During our yeast two-hybrid screen, we have isolated five other NCR-sensitive transcription activators and repressors (Dal80p, Dal81p, Dal82p, Gat1p, and Gz33p) that interacted with Tor1p (Table I). Gat1p appears to be responsible for the expression of GAP1 and GLN1 in the absence of Gln3p (18). We have also carried out a genome-wide expression profiling analysis of yeast cells treated with rapamycin. In agreement with TOR as a major regulator of transcription of NCR-sensitive genes, many such genes were indeed regulated by TOR (Table II and Ref. 17).

Because TOR is a protein kinase and interacts with Gln3p, we asked whether the kinase domain of TOR is required for the repression of the Gln3p-dependent genes. We performed Northern blotting analysis of GAP1 and GLN1 in cells carrying a plasmid expressing the recombinant wild type Tor1p or dominant rapamycin-resistant Tor1p(S1972I) in the wild type strain. Both cells also express the endogenous, wild type Tor1p and Tor2p. FKBP12-rapamycin only binds to the recombinant, wild type Tor1p as well as the endogenous wild type Tor1p and Tor2p but not the Tor1p mutants with the S1972I in the FKBP12-rapamycin-binding domain. The bulky isoleucine residue prevents the binding of FKBP12-rapamycin to the hydrophobic pocket of the FKBP12-rapamycin-binding domain (11, 26, 27). We found that rapamycin failed to cause the expression of GAP1 and GLN1 in cells producing recombinant Tor1p(S1972I) (Fig. 2c). Thus, the ability of rapamycin to induce the expression of GAP1 and GLN1 is TOR-dependent. We similarly studied the importance of the kinase domain of Tor1p using yeast expressing the kinase-dead Tor1p(S1972D2294E). D2294E is a mutation in the kinase domain that disables Tor1p kinase (8, 11). Unlike the cells expressing Tor1p(S1972I), rapamycin caused an induction of GAP1 and GLN1 in the Tor1p(S1972D2294E) cells, even though FKBP12-rapamycin is incapable of binding to both forms of Tor1p (Fig. 2c). Thus, an intact kinase domain or the kinase activity of Tor1p is required for repressing Gln3p-dependent genes.

To examine how Gln3p is regulated by TOR signaling, we have generated Gln3p tagged with Myc epitopes on the C terminus of the chromosomal copy of GLN3. Both rapamycin and nitrogen starvation caused rapid cytoplasm-to-nucleus translocation of Gln3p-Myc (18). An analog drug FK506 or the drug carrier methanol did not have the same effect. Thus, Gln3p is normally sequestered in the cytoplasm in the nitrogen-rich conditions. When cells are starved or treated with rapamycin, Gln3p translocated into the nucleus to activate the expression of NCR-sensitive genes (Fig. 3, a and b). In contrast, Tor1p (data not shown) remained in the cytoplasm in the presence of rapamycin, suggesting that the regulation of Gln3p primarily occurs in the cytoplasm. To analyze whether the rapamycin effect is dependent on TOR and its kinase activity, we expressed recombinant wild type Tor1p, rapamycin-resistant Tor1p(S1972I) or the kinase-inactive Tor1p(S1972I),...
the combination of Gal4p BD fusions and Gal4p AD fusions were
Ure2p in the yeast two-hybrid assay. Yeast PJ69–4a strains expressing
cally and genetically.

were separated by SDS-polyacrylamide gel electrophoresis and detected
by Western blotting with anti-Myc mAb 9E10.

were expressed in yeast and assayed for their ability to interact
with both Gln3p and Ure2p. The Gal4p BD and different Tor1p fusion
proteins were expressed in yeast and assayed for their ability to interact
with Tor1p-Myc9. GST (lane 1), the N-terminal 1–1764aa fragment of Tor1p is sufficient to interact
Tor2p(aa131–2475) and AD-Gln3p(aa510–720); 2, BD-Tor1p and
Ure2p(aa93–354); 3, BD-Tor1p-Myc and AD-Gln3p(aa510–720); 4, BD-Tor2p(aa131–2475) and
AD-Gln3p(aa510–720); 5, BD-Tor1p and Ure2p(aa93–354); 6, BD-Tor2p(aa131–2475) and Ure2p(aa93–354). b, upper panel, Tor1p binds to Gln3p and Ure2p in vitro. Extracts of yeast expressing Tor1p-Myc9 were incubated with GST (lane 1), GST-Gln3p(aa510–720) (lane 2), or GST-Ure2p(aa93–354) (lane 3) bound to glutathione-agarose beads. GST fusion proteins and bound materials were separated by SDS-polyacrylamide gel electrophoresis and detected by Western blotting with anti-Myc mAb 9E10. Total, total input of Tor1p-Myc9. Lower panel, GST and GST fusion proteins used for binding to Tor1p-Myc9, GST (lane 1), GST-Gln3p(aa510–720) (lane 2), and GST-Ure2p(aa93–354) (lane 3) were separated on SDS-polyacrylamide gel and stained with Coomassie Blue. MWM, molecular weight marker. c, the N-terminal 1–1764aa fragment of Tor1p is sufficient to interact with both Gln3p and Ure2p. The Gal4p BD and different Tor1p fusion proteins were expressed in yeast and assayed for their ability to interact with AD-Gln3p(aa510–720) and Ure2p(aa93–354). + indicates binding; − shows no binding. d, the Δgln3 and Δure2 mutations cause dramatic rapamycin sensitivities. Wild type and mutant yeast cells were streaked onto YPD or YPD plus rapamycin plates and incubated at 30 °C. WT, wild type yeast; RR, rapamycin-resistant strain with the TOR/S1972I mutation (11).

FIG. 1. TOR interacts with Gln3p and Ure2p both biochemi-
cally and genetically. a, Tor1p and Tor2p interact with Gln3p and
Ure2p in the yeast two-hybrid assay. Yeast PJ69–4a strains expressing
the combination of Gal4p BD fusions and Gal4p AD fusions were
streaked on SC-Leu · Trp · and SC-Leu · Trp · Ade · plates and incubated for 2 days. 1, BD and AD-Gln3p(aa510–720); 2, BD and AD-Ure2p(aa93–254); 3, BD-Tor1p and AD-Gln3p(aa510–720); 4, BD-Tor2p(aa131–2475) and AD-Gln3p(aa510–720); 5, BD-Tor1p and
Ure2p(aa93–354); 6, BD-Tor2p(aa131–2475) and Ure2p(aa93–354). b, upper panel, Tor1p binds to Gln3p and Ure2p in vitro. Extracts of yeast expressing Tor1p-Myc9 were incubated with GST (lane 1), GST-Gln3p(aa510–720) (lane 2), or GST-Ure2p(aa93–354) (lane 3) bound to glutathione-agarose beads. GST fusion proteins and bound materials were separated by SDS-polyacrylamide gel electrophoresis and detected by Western blotting with anti-Myc mAb 9E10. Total, total input of Tor1p-Myc9. Lower panel, GST and GST fusion proteins used for binding to Tor1p-Myc9, GST (lane 1), GST-Gln3p(aa510–720) (lane 2), and GST-Ure2p(aa93–254) (lane 3) were separated on SDS-polyacrylamide gel and stained with Coomassie Blue. MWM, molecular weight marker. c, the N-terminal 1–1764aa fragment of Tor1p is sufficient to interact with both Gln3p and Ure2p. The Gal4p BD and different Tor1p fusion proteins were expressed in yeast and assayed for their ability to interact with AD-Gln3p(aa510–720) and Ure2p(aa93–354). + indicates binding; − shows no binding. d, the Δgln3 and Δure2 mutations cause dramatic rapamycin sensitivities. Wild type and mutant yeast cells were streaked onto YPD or YPD plus rapamycin plates and incubated at 30 °C. WT, wild type yeast; RR, rapamycin-resistant strain with the TOR/S1972I mutation (11).
vented the gel mobility increase of Gln3p by rapamycin. Taken together, our results show that an intact TOR kinase is required for the \textit{in vivo} phosphorylation and inhibition of nuclear translocation of Gln3p, and the expression of Gln3p-dependent genes. To ask whether Tor1p can directly phosphorylate Gln3p, we incubated purified wild type and kinase-dead mutant Tor1p with bacterially expressed Gln3p in the presence of $[^{32}\text{P}]\text{ATP}$. We found that Gln3p was specifically phosphorylated by the wild type Tor1p but not the kinase-dead Tor1p(D2294E) (Fig. 4c). An overlay of the $^{32}\text{P}$-phosphorylated Gln3p and the Ponceau Red-stained Gln3p indicated that there was a gel mobility retardation associated with the phosphorylated Gln3p (data not shown), suggesting that TOR is responsible for Gln3p phosphorylation \textit{in vivo}. Further identification of the rapamycin-sensitive phosphorylation site(s) on Gln3p will be important to confirm that TOR is the relevant Gln3p kinase.

Activation of Gln3p requires dephosphorylation by protein phosphatase(s). There has been a controversy regarding the role of Tap42p\textsuperscript{z} Sit4p. Beck and Hall (18) reported that Tap42p\textsuperscript{z} Sit4p is crucial for the activation of Gln3p, whereas Cardenas \textit{et al.} (19) concluded that it is not required. We have

---

**TABLE I**

\textbf{Interaction of Tor1p with NCR-sensitive transcription factors}

These results are based on the yeast two-hybrid interaction assay. AD, Gal4p activation domain; BD, Gal4p DNA-binding domain; $^+$, the relative interaction strength; 0, no interaction; ND, not determined.

|       | BD   | BD-Tor1p | BD-HEAT | BD-p53 | BD-LamC |
|-------|------|----------|---------|--------|--------|
| AD    | 0    | 0        | 0       | 0      | 0      |
| AD-SV40 1\textsuperscript{T} | 0    | ND       | ND      | $^+$    | 0      |
| AD-Gln3p | 0    | $^+$     | $^+$    | 0      | 0      |
| AD-Ure2p | 0    | $^+$     | $^+$    | 0      | 0      |
| AD-Da180p | 0    | $^+$     | $^+$    | 0      | 0      |
| AD-Da181p | 0    | $^+$     | $^+$    | 0      | 0      |
| AD-Da182p | 0    | $^+$     | $^+$    | 0      | 0      |
| AD-Gat1p | 0    | $^+$     | $^+$    | 0      | 0      |
| AD-Gzf3p | 0    | $^+$     | $^+$    | 0      | 0      |

**TABLE II**

\textbf{Rapamycin-inducible, TOR-dependent NCR-sensitive genes by the yeast genome-wide expression profiling analysis (with an induction of 3-fold or more)}

| Open reading frame | Folds of induction | Description of gene function |
|-------------------|--------------------|-----------------------------|
|                   | 10 min | 30 min|
| AGP1              | 8.7    | 15.3 | Broad range amino acid permease |
| CAN1              | 4.9    | 5.5  | Permease for basic amino acids |
| DAL5              | 2.0    | 6.5  | Allantoate and ureidosuccinate permease |
| DIP5              | 7.1    | 9.9  | Dicarboxylic permease |
| DUR3              | 11.4   | 23.9 | Urea permease |
| GAP1              | 70.3   | 118.7 | General amino acid permease |
| MFP2              | 24.2   | 47.1 | Ammonia permease |
| UGA4              | 1.4    | 4.2  | $\gamma$-Aminobutyric acid permease |
| APE2              | 1.4    | 3.0  | Aminopeptidase II |
| ARG1              | 1.1    | 2.6  | Argininosuccinate synthetase |
| ARG4              | 1.3    | 10.9 | Argininosuccinate lyase |
| ARG10             | 1.3    | 3.6  | Ornithine acetyltransferase |
| AR09              | 1.2    | 9.0  | Aromatic amino acid aminotransferase |
| ASF3              | 1.2    | 2.8  | $\alpha$-Asparaginase II |
| BAT2              | 5.4    | 12.2 | Branched chain amino acid transaminase |
| CAR1              | 3.4    | 8.4  | Arginase |
| CPS1              | 3.4    | 19.2 | Carboxypeptidase |
| DAL2              | 3.6    | 10.8 | Allantoicase |
| DAL3              | 2.8    | 5.8  | Ureidoglycolate hydrolase |
| DAL7              | 2.0    | 6.5  | Malate synthase 2 |
| GDH1              | 3.3    | 6.7  | Glutamate dehydrogenase (NADH$^+$) |
| GDH2              | 14.1   | 8.4  | Glutamate dehydrogenase 2 |
| LYS1              | 1.3    | 4.3  | Saccharopine dehydrogenase |
| LYS21             | 1.0    | 2.7  | Homocitrurate synthetase isoenzyme |
| UGA1              | 2.6    | 3.4  | $\gamma$-Aminobutyric acid transaminase |
| UGA3              | 1.7    | 5.3  | Transcription factor for $\gamma$-aminobutyric acid catabolite genes |
| YBR139w           | 5.9    | 17.5 | Similar to serine type carboxypeptidase |
| YDL214c           | 7.9    | 4.2  | Protein kinase similar to NPR1 |
| YDR380w           | 1.2    | 6.4  | Putative indole-3-pyruvate decarboxylase |
| YFR055w           | 4.9    | 8.2  | Similar to cystathionine $\beta$-lyase |

**FIG. 3.** An intact kinase domain of TOR is required for regulating the nuclear entry of Gln3p. a, rapamycin causes rapid nuclear entry of Gln3p. Exponentially growing yeast cells expressing Gln3p-Myc\textsuperscript{9} in YPD were treated with a drug carrier methanol, 200 nM rapamycin, or FK506 for 10 min. The localization of Gln3p-Myc\textsuperscript{9} was examined by indirect immunofluorescence staining with mAb 9E10. The nucleus was stained with 4,6-diamidino-2-phenylindole. The magnification is 1000 x. b, nitrogen nutrients regulate the cytoplasm versus nuclear localization of Gln3p. Exponentially growing yeast cells expressing Gln3p-Myc\textsuperscript{9} were switched from the nitrogen complete (1\textsuperscript{N}) medium to the nitrogen-deficient (2\textsuperscript{N}) medium. c, TOR regulates the nuclear entry of Gln3p by phosphorylation. Yeast carrying a vector control, or expressing Tor1p(S1972I) or Tor1p(S1972I, D2294E) were treated with 200 nM rapamycin for 10 min.
The role of Ure2p in the regulation of Gln3p.

a, Gln3p mediates the binding of Ure2p to the TOR complex. The interaction between BD-Tor1p and AD-Ure2p requires endogenous Gln3p. Yeast strains expressing the combination of Gal4p BD fusions and Gal4p AD fusions were streaked on SC-Leu<sup>−</sup> Trp<sup>−</sup> Ade<sup>−</sup> and SC-Leu<sup>−</sup> Trp<sup>−</sup> plates and incubated for 2 days. Lanes 1–4, assays were performed in the Δgln3 strain; lanes 5–8, assays were performed in the Δure2 strain. Lane 1, BD and AD-Ure2p(aa93–254); lane 2, BD-Tor1p and Ure2p(aa93–354); lane 3, BD and AD-SV40-LgT (negative control); lane 4, BD-p53 and AD-SV40-LgT (positive control); lane 5, BD and AD-Gln3p(aa510–720); lane 6, BD-Tor1p and AD-Gln3p(aa510–720); lane 7, BD and AD-SV40-LgT (negative control); lane 8, BD-p53 and AD-SV40-LgT (positive control).

b, rapamycin reduces the Gln3p Ure2p complex in vivo. Extracts of yeast cells expressing Gln3p-Myc<sup>9</sup> alone, or together with Ure2p tagged with protein A (Ure2p-ProA) cultured in the presence or absence of 200 nM rapamycin were prepared. Ure2p-ProA was immunoprecipitated with IgG-Sepharose beads. Ure2p-ProA and its associated Gln3p-Myc<sup>9</sup> were detected by Western blotting with mAb 9E10.

c, Ure2p binds to both phosphorylated and dephosphorylated Gln3p. Extracts of exponential yeast cells expressing Gln3p-Myc<sup>9</sup> were treated with CIP in the absence or presence of Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> and incubated with immobilized, bacterially produced GST-Ure2p or GST. Upper panel, Western blot with anti-Myc mAb 9E10; lower panel, Coomassie Blue staining of the GST-Ure2p and GST used.

d, Gln3p bound to Ure2p is resistant to phosphatase. Extracts of yeast expressing Gln3p-Myc<sup>9</sup> and Ure2p-ProA were incubated without or with CIP. Ure2p-ProA and bound materials were affinity-purified with IgG-Sepharose and examined by Western blotting with mAb 9E10.
also examined whether activation of Gln3p-dependent genes by rapamycin is affected by the tap42-11 mutation. Tap42p is the regulatory subunit of the type 2 protein phosphatase Sit4p (29). tap42-11 is a gain-of-function mutation that confers rapamycin resistance at permissive temperature in polysomal formation (29) and dephosphorylation of Npr1p (30). At a low concentration of rapamycin (25 nM), the induction of GAP1 was attenuated in the tap42-11 mutant strain at the permissive temperature (Fig. 4d). However, this attenuation by the tap42-11 mutation gradually diminished with increasing rapamycin concentrations (data not shown). Thus, the difference between the two previous observations could attribute to different rapamycin concentrations used in their respective experiments. Although Tap42p-Sit4p appears to be involved in the activation of Gln3p, it may only play a redundant role with other phosphatases. Indeed, we also found that the expression of GAP1 by rapamycin was similarly attenuated in cells lacking Pph3p, another general protein phosphatase (Fig. 4e). It should be noted that we do not have evidence that Sit4p and Pph3p are directly responsible for the dephosphorylation of Gln3p.

To examine the role of Ure2p in the regulation of Gln3p by TOR, we examined the interaction among TOR, Gln3p, and Ure2p. Although Gln3p still interacted with Tor1p without Ure2p, Ure2p failed to bind to Tor1p in the absence of Gln3p (Fig. 5a). Thus, the interaction between Ure2p and TOR is mediated by Gln3p. As previously reported (18), rapamycin reduced the amount of Gln3p bound to Ure2p (Fig. 5b). In contrast to that study, however, we found that the bacterially expressed GST-Ure2p bound to the hyper-phosphorylated Gln3p and the dephosphorylated Gln3p generated by CIP treatment in vitro (Fig. 5c) or rapamycin treatment in vivo (data not shown). In fact, we have consistently observed that the dephosphorylated Gln3p has a higher affinity for Ure2p (Fig. 5c). Gln3p also remained bound to Ure2p in both whole cell extracts (Fig. 5d) and purified Ure2p-immunocomplex treated with CIP (data not shown). Thus, it seems unlikely that phosphorylation regulates the interaction between Ure2p and Gln3p. Instead, the Ure2p-bound Gln3p is considerably more resistant to CIP treatment than Ure2p and the free Gln3p (Fig. 5d). Therefore, the binding of Ure2p appears to protect Gln3p from dephosphorylation, which is also consistent with a previous observation that overexpression of Ure2p causes accumulation of slower gel mobility forms of Gln3p (28).

**DISCUSSION**

In this study, we show that TOR binds to Gln3p, which is mediated by the HEAT domain of TOR. We further show that an intact kinase domain is required for the phosphorylation of Gln3p as well as repression of Gln3p cytoplasm-to-nucleus translocation and Gln3p-dependent transcription. At least two protein phosphatases, Tap42p-Sit4p and Pph3p are involved in the activation of GAP1, a gene regulated by Gln3p. Ure2p binds to both hyper- and hypo-phosphorylated Gln3p. However, it appears to protect Gln3p from dephosphorylation. Taken together, our results reveal a tripartite regulation of phosphorylation of Gln3p (a model shown in Fig. 6). Perturbation of the delicate balance among the three factors such as rapamycin treatment or deletion of URE2 results in a deregulated Gln3p. An important challenge in the near future is to determine how nutrient signals alter such a balance.

In addition to Gln3p and Ure2p, five other NCR-sensitive transcription regulators (Gat1p, Gzf3p, Dal80p, Dal81p, and Dal82p) also interact with Tor1p and Tor2p (Table I). These proteins are known to serve as transcriptional activators as well as repressors. They regulate diverse NCR-sensitive genes in response to different nitrogen nutrients such as amino acids, allantoin, ammonia, and urea. Such interactions imply that TOR also regulates these NCR-sensitive transcription regulators. Similarly, a wide variety of NCR-sensitive genes are rapidly responsive to rapamycin as indicated by gene expression profiling studies (Table II and Refs. 17 and 19). Therefore, combinatorial regulation of different NCR-sensitive transcription regulators by TOR is likely to be responsible for the well orchestrated expression of NCR-sensitive genes in response to specific nitrogenous compounds.

The N terminus of TOR contains 20 HEAT repeats. HEAT repeats form elongated coiled coil structures as indicated by the crystal structures of Karyopherin α and the A subunit of type 2 protein phosphatase. Although the secondary structures of the HEAT repeats are highly conserved, their primary sequences bear a very low degree of similarity. The primary role of the HEAT repeats has been suggested to mediate protein-protein interaction. However, the protein partners appear to be different for individual HEAT repeat-containing proteins. In agreement with this hypothesis, we found that the HEAT region of Tor1p is necessary and sufficient to bind to multiple proteins, including Gln3p and Gat1p (Table I). A recent study also shows that the same region of RAFT (mammalian TOR) also interacts with gelsyn, a cytoskeletal protein necessary for RAFT signaling to p70S6 kinase (31). These results show that the HEAT repeats of TOR are involved in protein-protein interactions as are other HEAT repeat-containing proteins.

The second important domain of TOR in the regulation of Gln3p is the kinase domain. Although the kinase domain of TOR shares significant similarity with the catalytic domains of phosphatidylinositol 3'-OH kinases, it only appears to have protein serine/threonine kinase activity. A number of rapamycin-sensitive proteins including 4EBP-1/Phas-1 and p70S6 kinase have been shown to be phosphorylated by TOR in vitro as...
well. In this study, we found that an intact kinase domain of TOR is critical for the in vivo phosphorylation and cytoplasmic retention of Gln3p as well as the repression of Gln3p-dependent genes. It is also required for Tor1p to phosphorylate Gln3p in vitro. These results show that the kinase activity of TOR plays an essential role to regulate Gln3p and suggest that TOR is directly responsible for phosphorylating Gln3p in vivo. The rapamycin-sensitive phosphorylation sites need to be identified to establish the relevance of Gln3p phosphorylation in its regulation and the role of TOR. In addition, TOR may phosphorylate Gln3p as well as inhibit the dephosphorylation of Gln3p by negatively regulating the phosphatases involved. However, whether Tap42pSit4p and Pph3p are directly involved in Gln3p dephosphorylation remains to be determined.

We found that Ure2p binds to both hyperphosphorylated and dephosphorylated forms of Gln3p. The bacterially recombinant GST-Ure2p binds to both hyper- and hypo-phosphorylated Gln3p in vitro (Fig. 5c). The hypo-phosphorylated Gln3p appears to have a slightly higher affinity to Ure2p. In addition, treatment of the purified Ure2p-Gln3p complex with phosphatase does not cause dissociation of Gln3p from Ure2p. Thus, the phosphorylation status of Gln3p does not appear to affect the binding of Ure2p. Instead, our results indicate that Ure2p protects Gln3p from dephosphorylation, which is also supported by a previous observation that overexpression of Ure2p leads to accumulation of hyper-phosphorylated Gln3p (28). How does Ure2p become rapidly dissociated from Gln3p upon rapamycin treatment or nitrogen starvation? Mks1p was genetically identified as an inhibitor of Ure2p (32, 33). Ure2p is also a phosphoprotein (Fig. 5 and Refs. 17 and 19). It becomes dephosphorylated in as a result of Ure2p treatment. Mks1p may preferentially bind to the dephosphorylated Ure2p, which prevents Ure2p from binding to Gln3p. In addition, the dephosphorylated Gln3p may be preferentially imported into the nucleus, thereby causing the equilibrium shifting toward fewer Ure2p-Gln3p complexes (a model is shown in Fig. 6).

Phosphorylation is broadly involved in the regulated transport of many important regulatory proteins between the cytoplasm and nucleus (34). Phosphorylation within or adjacent to the nuclear localization signal sequence can dramatically affect recognition of cargo proteins by the nuclear importins. One such example is Pho4p, a phosphate-regulated transcription factor. Pho4p is phosphorylated and localized in the cytoplasm when yeast is grown in phosphate-rich medium, whereas in low phosphate medium, Pho4p is dephosphorylated and localized to the nucleus. Pho4p nuclear translocation required an importin called Pse1p. Phosphorylation decreases the affinity of Pho4p to Pse1p, thereby causing the cytoplasmic retention of Pho4p (35). A similar scenario could also apply to Gln3p and other NCR-sensitive transcription activators and repressors (a model is shown in Fig. 6). Identification of the nuclear importin and exportin for Gln3p will be important to further define the mechanism by which the nucleocytoplasmic transport of Gln3p is regulated.

Acknowledgments—We are grateful to D. Dean for critical reading of this manuscript, K. Arndt, R. Brachmann, C. Hardy, P. James, M. Johnston, and S. Wente for strains, plasmids and libraries, C. He for work involved in the yeast two-hybrid screens, and A. Chu for mAb 9E10.

REFERENCES
1. Magasanik, B. (1992) in The Molecular and Cellular Biology of Yeast Saccharomyces, Vol. 2, pp. 283–318, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
2. Taylor, K. L., Cheng, N., Williams, R. W., Steven, A. C., and Wickner, R. B. (1999) Science 283, 1399–1439.
3. Coffman, J. A., Rai, R., Logrete, D. M., Cunningham, T., Svetlov, V., and Cooper, T. G. (1997) J. Bacteriol. 179, 3416–3429.
4. Coomert, D., Vissers, S., and André, B. (1991) Gene (Amst.) 97, 163–171.
5. Heitman, J., Murov, N. R., and Hall, M. N. (1991) Science 253, 905–909.
6. Cooper, K., Maclaughlin, M. M., Young, P. R., Johnson, R. K., and Livi, G. P. (1994) Gene (Amst.) 141, 133–136.
7. Hellwell, S. B., Wagner, P., Kunz, J., Deuter-Reinhard, M., Henriquez, R., and Hall, M. N. (1994) Mol. Biol. Cell 5, 105–118.
8. Bertram, P. G., Zeng, C., Thorsen, J., Shaw, A. S., and Zheng, X. F. (1998) Curr. Biol. 8, 1259–1267.
9. Jiang, Y., and Brasch, J. R. (1999) EMBO J. 18, 2782–2792.
10. Aracnon, C. M., Heitman, J., and Cardenas, M. E. (1999) Mol. Biol. Cell 10, 2531–2546.
11. Zheng, X. F., Florentino, D., Chen, J., Crabtree, G. R., and Schreiber, S. L. (1995) Cell 82, 121–130.
12. Dennis, P. B., Fumagalli, S., and Thomas, G. (1999) Curr. Opin. Genet. Dev. 9, 49–54.
13. Kurnavilla, F., and Schreiber, S. L. (1999) Chem. Biol. 6, R129–R136.
14. Mahajan, P. B. (1994) Int. J. Immunopharmacol. 16, 711–721.
15. Zaraqua, G., Ghavidel, A., Heitman, J., and Schach, M. C. (1998) Mol. Cell. Biol. 18, 4436–4447.
16. Power, T., and Walter, P. (1999) Mol. Biol. Cell 10, 987–1000.
17. Hardwick, J. S., Kurnavilla, F. G., Tong, J. K., Shimoni, A. F., and Schreiber, S. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 14866–14870.
18. Beck, T., and Hall, M. N. (1999) Nature 402, 689–692.
19. Cardenas, M., Cutler, N., Lorenz, M., Di Como, C., and Heitman, J. (1999) Genes Dev. 13, 3217–3227.
20. James, P., Halladay, J., and Craig, E. A. (1996) Gene 178, 115–132.
21. Schneider, B. L., Seufert, W., Steiner, B., Yang, Q. H., and Butcher, A. B. (1995) Yeast 11, 1285–1274.
22. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hiufer, P., and Boeke, J. D. (1998) Yeast 14, 115–132.
23. Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1999) Nucleic Acids Res. 18, 3991–3992.
24. Adams, A., Gottschling, D. E., Kaiser, C. A., and Stearns, T. (1997) Methods in Yeast Genetics, pp. 137–137, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Andrade, M. A., and Bork, P. (1995) Nat. Genet. 11, 115–116.
26. Chen, J., Zheng, X. F., Brown, E. J., and Schreiber, S. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4947–4951.
27. Cho, J., Chen, J., Schreiber, S. L., and Claridy, J. (1998) Science 273, 239–242.
28. Blinder, D., Cochiogno, P. W., and Magasanik, B. (1996) J. Bacteriol. 178, 4734–4736.
29. Di Como, C. J., and Arndt, K. T. (1996) Genes Dev. 10, 1904–1916.
30. Schmidt, A., Beck, T., Keller, A., Kunz, J., and Hall, M. N. (1998) EMBO J. 17, 6924–6931.
31. Sabatini, D. M., Barlow, R. K., Blackshaw, S., Burnett, P. E., Lai, M. M., Field, M. E., Bahr, B. A., Kirsch, J., Betz, H., and SH., S. (1999) Science 284, 1161–1164.
32. Edkshe, K. H., Hannover, J. A., and Wickner, R. B. (1999) Genetics 153, 585–594.
33. Edkshe, K. H., and Wickner, R. B. (2000) Proc. Natl. Acad. Sci. U. S. A. 107, 3085–3089.
34. Hood, J. K., and Silver, P. A. (1999) Curr. Opin. Cell Biol. 11, 241–247.
35. Kaffman, A., Rank, N. M., O'Neill, E. M., Huang, L. S., and O'Shea, E. K. (1998) Nature 396, 482–486.