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TOR Controls Translation Initiation and Early G1 Progression in Yeast

Nik C. Barbet,* Ulrich Schneider,* Stephen B. Helliwell,* Ian Stansfield,† Michael F. Tuite,† and Michael N. Hall*‡

*Department of Biochemistry, Biozentrum, University of Basel, CH-4056 Basel, Switzerland; and †Research School of Biosciences, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ, United Kingdom

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Saccharomyces cerevisiae cells treated with the immunosuppressant rapamycin or depleted for the targets of rapamycin TOR1 and TOR2 arrest growth in the early G1 phase of the cell cycle. Loss of TOR function also causes an early inhibition of translation initiation and induces several other physiological changes characteristic of starved cells entering stationary phase (G0). A G1 cyclin mRNA whose translational control is altered by substitution of the *UBI4* 5′ leader region (UBI4 is normally translated under starvation conditions) suppresses the rapamycin-induced G1 arrest and confers starvation sensitivity. These results suggest that the block in translation initiation is a direct consequence of loss of TOR function and the cause of the G1 arrest. We propose that the TORs, two related phosphatidylinositol kinase homologues, are part of a novel signaling pathway that activates eIF-4E-dependent protein synthesis and, thereby, G1 progression in response to nutrient availability. Such a pathway may constitute a checkpoint that prevents early G1 progression and growth in the absence of nutrients.

INTRODUCTION

The immunosuppressant rapamycin and the related compound FK506 exert their immunosuppressive effects by inhibiting intermediate steps in signal transduction that lead to T cell activation and proliferation (Heitman *et al.*, 1991; Schreiber and Crabtree, 1992; Sigal and Dumont, 1992; Liu, 1993; Fruman *et al.*, 1994). FK506 in complex with its intracellular receptor FKBP inhibits the Ca^{2+}/calmodulin-dependent phosphatase calcineurin. As a downstream effector of the T cell receptor (TCR), calcineurin normally triggers nuclear import of a subunit of the transcription factor NF-AT which, in turn, activates 50–100 genes, including the gene encoding the lymphokine interleukin-2 (IL-2) (Weiss and Littman, 1994). Rapamycin also forms a toxic complex with FKBP, but instead of inhibiting the TCR signaling pathway, inhibits a subsequent signal transduction cascade that is stimulated by IL-2 (Bierer *et al.*, 1990; Dumont *et al.*, 1990). The IL-2 signaling pathway mediates G1 progression (proliferation) of a T cell. Rapamycin prevents the phosphorylation and activation of p70 S6 kinase, a downstream effector of IL-2 and several other growth factors, including insulin, EGF, PDGF, IL-3, and erythropoietin (Calvo *et al.*, 1992, 1994; Chung *et al.*, 1992; Kuo *et al.*, 1992; Price *et al.*, 1992; Terada *et al.*, 1992; Ferrari *et al.*, 1993; Lane *et al.*, 1993). Although best known for its inhibition of IL-2–dependent p70 S6 kinase activation, rapamycin also inhibits p70 S6 kinase activation in response to these other mitogens (Calvo *et al.*, 1992; Chung *et al.*, 1992, 1994; Price *et al.*, 1992). The p70 S6 kinase phosphorylates the ribosomal protein S6 which, in turn, leads to the activation of translation initiation (Kuo *et al.*, 1992; Thomas, 1992; Jefferies *et al.*, 1994; Terada *et al.*, 1994). The p70 S6 kinase thus links mitogenic stimulation and the initiation of protein synthesis. A homologue of the yeast TOR proteins (FRAP/RAFT1/RAPT1/mTOR) (see below) has recently been identified in mammalian cells as a direct target of the rapamycin-FKBP complex (Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995), suggesting that FRAP/RAFT1/RAPT1/mTOR is required for p70 S6 kinase activation and is part of a general mitogenic signaling

‡ Corresponding author.

pathway (for a figure that summarizes the pathway, see Downward, 1994).

In the yeast *Saccharomyces cerevisiae*, rapamycin-FKBP inhibits the *TOR1* and *TOR2* gene products and arrests cells with an unbudded morphology, indicative of a G1 cell cycle arrest similar to that observed in T cells (Heitman *et al.*, 1991; Cafferkey *et al.*, 1993; Kunz and Hall, 1993; Kunz *et al.*, 1993; Helliwell *et al.*, 1994; Stan *et al.*, 1994; Zheng *et al.*, 1995). A dominant point mutation in either *TOR* gene renders a cell resistant to rapamycin, whereas disruption of both genes results in an unbudded morphology, similar to that seen when wild-type cells are treated with rapamycin, and a 1n DNA content (Heitman *et al.*, 1991; Cafferkey *et al.*, 1993; Kunz *et al.*, 1993; Helliwell *et al.*, 1994). TOR1 and TOR2 are large (~280 kDa), functionally homologous proteins that are structurally related to phosphatidylinositol kinases (PI kinases) (Kunz *et al.*, 1993; Garcia-Bustos *et al.*, 1994; Helliwell *et al.*, 1994; Yoshida *et al.*, 1994). PI kinases are required for production of phosphatidylinositol-derived second messengers (Carpenter and Cantley, 1990). Thus, TOR1 and TOR2, like FRAP/RAFT1/RAPT1/mTOR, may be components of a rapamycin-sensitive signaling pathway required for cell cycle progression in G1.

In *S. cerevisiae*, the decision to commit to a cell cycle is made at a point in late G1 termed START. Traversal of START and entry into S phase is regulated by the activity of the cyclin-dependent kinase encoded by *CDC28* (Reed, 1992; Nasmyth, 1993). Three G1-specific cyclin genes were originally identified and named *CLN1*, *CLN2*, and *CLN3* (Cross, 1988; Nash *et al.*, 1988; Richardson *et al.*, 1989; Wittenberg *et al.*, 1990); additional candidate G1 cyclin genes have subsequently been identified and named *HCS26*, *ORFD*, *CLB5*, and *CLB6* (Frohlich *et al.*, 1991; Ogas *et al.*, 1991; Epstein and Cross, 1992; Kuehne and Linder, 1993; Schwob and Nasmyth, 1993). All except *CLN3* are transcribed only in late G1 with *CLN1*, *CLN2*, and *HCS26*, and possibly *ORFD*, under control of the transcription factor SBF. CLN3, whose transcript is present throughout the cell cycle, is regulated post-transcriptionally and acts as an upstream activator of other G1 cyclins (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991; Tyers *et al.*, 1992, 1993; Cvrckova and Nasmyth, 1993).

When nutrients are limiting, haploid yeast cells do not proceed to START in late G1, but instead exit the mitotic cell cycle in early G1 and enter a stationary or G0 phase (for review see Werner-Washburne *et al.*, 1993). Stationary phase enables a cell to maintain viability for long periods when nutrients are not available, and is characterized by several physiological properties including 1n DNA content, failure to reach START, reduced protein synthesis, accumulation of glycogen, acquisition of thermotolerance, and changes in the pattern of transcription (Werner-Washburne *et al.*, 1993). Nutrient sensing and the regulation of entry into stationary phase are poorly understood, but are generally thought to involve the RAS/cAMP pathway (Broach, 1991; Thevelein, 1994). However, this is not the sole nutrient-sensing pathway, as mutants in the RAS/cAMP pathway have been isolated that exhibit a normal response to starvation independently of intracellular cAMP levels (Cameron *et al.*, 1988).

Here we report that cells lacking TOR function (cells treated with rapamycin or depleted of TOR) arrest growth and rapidly exhibit, by all criteria examined, properties diagnostic of G0 or stationary phase, including a reduction in translation initiation. The cell cycle arrest upon loss of TOR function is suppressed by altering the translational control of the G1 cyclin CLN3. Our results and analogy with mammalian cells suggest that TOR is part of a novel signal transduction pathway required for translation initiation and G1 progression, perhaps in response to nutrients.

MATERIALS AND METHODS

Strains, Plasmids, and Media

The parental strain in this study was JK9–3da (*MATa leu2–3, 112 ura3–52 trp1 his4 rme1 HMLa*). Isogenic derivatives with only the changes indicated are shown in Table 1. The composition of rich medium (YPD), synthetic galactose/glycerol medium (SGal/Gly), and synthetic glucose medium (SD) supplemented with the appropriate nutrients was as described (Sherman, 1991). All cultures were incubated at 30°C unless otherwise indicated. Rapamycin (provided by Sandoz Pharma, Basel, Switzerland) was added to the medium to a final concentration of 0.2 μg/ml. Rapamycin was diluted into media from a stock solution of 1 mg/ml in 10% Tween-20/90% ethanol (Heitman *et al.*, 1993). Plasmid pJK5 contains the entire *TOR2* gene under control of the *GAL1* promoter (Kunz *et al.*, 1993). YEplac181::*tor2–61*^{ts} (amp^{r} 2 μ *LEU2*) contains the entire *TOR2* gene and was isolated by hydroxylamine mutagenesis as a temperature-sensitive *TOR2* allele (Barbet and Hall, unpublished data). The *RAS2*^{val19} allele on plasmid YEp213 (amp^{r} 2 μ *URA3*) (Broek *et al.*, 1987) was transformed into JK9–3da. YCplac111 is amp^{r} *CEN4 LEU2* (Gietz and Sugino, 1988). The *BCY1* gene was disrupted (*bcy1::URA3*) as described using the one-step gene replacement technique (Toda *et al.*, 1987; Rothstein, 1991). Integration of *ADH-*

Table 1. Strains used in this study

Strain	Genotype
JK9-3da	*MATa leu2-3,112 ura3-42 trp1 his4 rme1 HMLa*
JK350-21a	JK9-3da *tor1:LEU2-4 tor2::ADE2-3*/pJK5
JH11-1c	JK9-3da *TOR1-1*
JH12-17b	JK9-3da *TOR2-1*
NB17-3d	JK9-3da *his3 HIS4 tor1::HIS3*
NB30	JK9-3da *bcy1::URA3*
NB32	JK9-3da *ura3::[URA3 ADH-CLN2]*
NB33	JK9-3da *ura3::[URA3 CLN3-1]*
NB34	JK9-3da/YEp213::*RAS2*^{val19}
NB35	JK9-3da *ade2 his3 HIS4 tor1::HIS3 tor2::ADE2*//YEplac181::*tor2-61*^{ts}
NB36	JK9-3da/YCplac111::*UB14*$^{5'}$*-CLN3*
NB37	JK9-3da/YCplac111::*UB14*$^{5'}$
NB38	JK9-3da/YEpURA::*CLN3*

CLN2 (Nasmyth and Dirick, 1991) at the *ura3* locus was achieved by linearizing the plasmid containing the *CLN2* construct with *Eco*RV. Disruptions and integrations were confirmed by Southern blot analysis. All transformations were performed using the lithium acetate procedure (Ito *et al.*, 1983).

Flow Cytometry

Overnight cultures of yeast in SD complete medium were diluted to OD_{600} <0.05 and allowed to grow before the addition of rapamycin at OD_{600} = 0.2. Three hundred-microliter samples were taken from these cultures at hourly intervals, sonicated for 2 min, and immediately fixed by addition of 700 μl absolute ethanol. Samples were incubated overnight at 4°C, washed, and resuspended in 50 mM sodium citrate, pH 7.4, and treated with RNAse (0.25 mg/ml) for 1 h at 37°C. DNA was stained by the addition of 500 μl citrate buffer containing 16 μg/ml propidium iodide. For each timepoint taken, 10,000 events were analyzed for DNA content using a Becton Dickinson FACScan (Mountain View, CA) and data was processed using Lysys II software (Lincoln Park, NJ).

Order-of-Function Mapping

A reciprocal shift experiment was performed with the temperature-sensitive *tor2* strain NB35 and α-factor as described (Hereford and Hartwell, 1974) and also with the modification of a 1.5-h overlap in which both blocks were imposed. Because the effects of rapamycin are irreversible (presumably because the drug cannot be washed out) we were unable to perform a standard reciprocal shift experiment with α-factor and rapamycin. To circumvent this problem, we performed a double block experiment and an α-factor to rapamycin shift experiment. For the double block experiment, logarithmically growing cultures of JK9–3da in SD medium were treated with nocodazole (10 μg/ml; Sigma, St. Louis, MO) for 2.5 h to arrest the cells in mitosis. Cells were harvested by filtration and nocodazole was washed out of the cells with 10 volumes of sterile water followed by 10 volumes of SD medium. Cells were then incubated in fresh SD medium either with no addition, with 10 μg/ml mating pheromone (α-factor), with 0.2 μg/ml rapamycin, or with both mating pheromone and rapamycin. Samples were taken at 30-min intervals, sonicated for 2 min to separate cells, and scored for emergence of buds and appearance of the shmoo phenotype (Sprague, 1991). For the α-factor to rapamycin shift experiment, logarithmically growing cultures of JK9–3da in SD medium were treated with 10 μg/ml α-factor for 2.5 h. Cells were harvested by filtration and washed with 10 volumes of sterile water followed by 10 volumes of SD medium. The culture was then split; one half of the culture received 0.2 μg/ml rapamycin and the other half received drug vehicle alone. Samples were removed at 30 min intervals, sonicated for 2 min, and scored for the emergence of buds.

Northern Analysis

Extraction of total cellular RNA was performed as previously described (Jensen *et al.*, 1983). For Northern analysis, 10 μg of total RNA was separated on 1% agarose gels containing 6% formaldehyde, and transferred overnight to Hybond-N+ nylon membrane (Amersham, Arlington Heights, IL) in 20× SSC. The *HCS26*, *ORFD*, *CTT1*, *SSA3*, *UBI4*, *CLB5*, and *CLN2* DNA probes were amplified from genomic DNA by the polymerase chain reaction (PCR). The primers used for PCR were as follows, with the 5′ primer listed first and the fragment size generated given in parentheses: *HCS26*, 5′-ATGTGTGAATACAGCAAG-3′ and 5′-AAACCCATGTTGACTCAT-3′ (963 bp); *ORFD*, 5′-ATGTCAAACTACGAAGCC-3′ and 5′-CCTGTGTTCTTCCGCCTT-3′ (998 bp); *CTT1*, 5′-ATGAACGTGTTCGGTAAA-3′ and 5′-TGGCACTTGCAATGGACC-3′ (1686 bp); *SSA3*, 5′-ATGTCTAGAGCAGTTGGT-3′ and 5′-ATCAACCTCTTCCACTGT-3′ (1947 bp); *UBI4*, 5′-ATGCAGATTTTCGTCAAG-3′ and 5′-GTTACCACCCCTCAACCT-3′ (1142 bp); *CLB5*, 5′-ATGGGAGAGAACCACGAC-3′ and 5′-TGCTATGCATTTCGGATG-3′ (1278 bp); and *CLN2*, 5′-ATGGCTAGTGCTGAACCA-3′ and 5′-TATTACTTGGGTATTGCC-3′ (1634 bp). The *SWI4* probe was a 2.2-kbp *Bam*HI fragment from the plasmid YCplac33::SWI4 (gift of K. Nasmyth). The *SWI6* probe was a 2.2-kbp *Xho*I/*Cla*I fragment from 1941 (gift of K. Nasmyth). The *CLN3* probe was a 500-bp *Hin*dIII/*Eco*RI fragment from pBF30 (Nash *et al.*, 1988). The probe for *CLN1* was a 2-kbp *Hin*dIII fragment from pcln1::URA3 (Hadwiger *et al.*, 1989). The probe for *CDC28* was a 1.2-kbp *Xho*I/*Xba*I fragment from YEp13::CDC28 (gift of K. Nasmyth). The probe for *TOR1* was a 4.3-kbp *Hin*dIII fragment from pPW20 (Helliwell *et al.*, 1994). The *TOR2* probe was a 5.3-kbp *Bgl*II fragment from pJK3–3 (Kunz *et al.*, 1993). The *HSP26* probe was a 800-bp *Bgl*II/*Nde*I fragment from pHSP26 (gift of S. Lindquist). The probe for *SSB1* was a 2.2-kbp *Hin*dIII fragment from pFKR15. The probe for *SSA1* was a 2-kbp *Sal*I fragment from EC551 (gift of E. Craig). The *ACT1* probe was a 1-kbp *Eco*RI/*Pst*I fragment from pUC18::*ACT1* (gift of P. Linder). *SSA1* and *SSA2* transcripts are indistinguishable, as are *SSB1* and *SSB2* transcripts, because the DNA sequences of these pairs of genes are 97% and 94% identical, respectively (Werner-Washburne *et al.*, 1989). Probes were labeled with [^{32}P]dATP using the random-primed DNA labeling kit (United States Biochemical, Cleveland, OH). Filters were exposed to x-ray film (Kodak X-OMAT) AR at −70°C with intensifying screens (Dupont Cronex). Signals were quantitated by scanning appropriately exposed films using a Molecular Dynamics densitometer (Sunnyvale, CA). In the experiment shown in Figure 7B, the total cellular RNAs of strains NB36 and NB38 were prepared identically, run on the same gel, transferred to the same filter, and hybridized to the same probe at the same time.

Incorporation of [^{35}S]Methionine into Total Yeast Protein

For analysis of gross protein synthesis, tricholoracetic acid (TCA)-precipitable counts were quantitated from pulse-labeled cultures at the indicated times after treatment. For rapamycin treatment, exponentially growing cultures of JK9–3da in SD medium minus methionine were treated with 0.2 μg/ml rapamycin, 100 μg/ml cycloheximide, or with drug vehicle alone (10% Tween-20/90% ethanol). For TOR depletion, exponentially growing cultures of the *tor*^{ts} strain NB35 and the control strain NB17–3d in SD minus methionine were resuspended in prewarmed medium at 37°C. For each timepoint, 0.01 OD_{600} equivalents were removed and labeled at 30°C for 7 min with 2 μCi [^{35}S]methionine (Amersham). Aliquots of the pulse-labeled cells were lysed on Whatman filters presoaked in 50% TCA, and deacylated by boiling for 10 min in 5% TCA. Filters were washed in acetone, air dried, and TCA-precipitable counts were quantitated by scintillation counting using a Canberra Packard 1900TR liquid scintillation analyzer.

Polysome Gradient Analysis

Strains JK9–3da and NB35 were grown in YPD to a cell density of 10^7 cells ml^{-1}. Following harvesting, polysomes were prepared as described (Stansfield *et al.*, 1992), except that polysomes were resolved on a 15–50% w/v sucrose gradient by centrifuging for 2.1 h at 17,000 × *g* using a Beckman SW40 Ti rotor. Cycloheximide (200 μg/ml) and rapamycin (0.2 μg/ml) were added to cultures at the indicated times before harvest. Drugs used in this way to inhibit yeast cultures were also included at the same concentration in the lysis buffer (Stansfield *et al.*, 1992).

Glycogen Staining

Logarithmically growing cultures in SD medium were treated with 0.2 μg/ml rapamycin and incubated at 30°C in the presence of the drug. At hourly intervals up to 5 h after rapamycin addition, 5 OD equivalents of cells were harvested onto Millipore HA filters (Bed-

ford, MA), placed upon a solid agar matrix, and exposed to iodine vapor for 1 min.

Construction and Analysis of the UBI4-CLN3 Fusion

The 5′ region (containing the untranslated leader and promoter sequences) of the *UBI4* polyubiquitin gene and a sequence containing the open reading frame of the *CLN3* gene were amplified from *S. cerevisiae* genomic DNA using the polymerase chain reaction. Oligonucleotides were designed to produce a 752-bp *UBI4* 5′ region fragment flanked by a 5′ *Hin*dIII and a 3′ *Sal*I restriction site, and a 1821-bp *CLN3* fragment flanked by 5′ *Sal*I and 3′ *Sma*I sites. The oligonucleotides were as follows: *UBI4* 5′ end, 5′-GCAAAGCTTC-CCACCACCAGCACTAGCTTAGAT-3′; *UBI4* 3′ end, 5′-AATGTC-GACCTATTAGTTAAAGTAAAGTGGGTG-3′; *CLN3* 5′ end, 5′-TACGTCGACTGTACGATGGCCATATTGAAGGAT-3′; and *CLN3* 3′ end, 5′-GTACCCGGGACGTATTTGCTTTGCAAATTTTA-3′. The *UBI4-CLN3* construct was obtained by first introducing the *Hin*dIII/*Sal*I-cut *UBI4* 5′ region fragment into a *Hin*dIII/*Sal*I cut YCplac111 vector (*CEN4 LEU2*). Following transformation and amplification in *E. coli*, this "parent plasmid" was digested with *Sal*I and *Sma*I and the *Sal*I/*Sma*I-cut *CLN3* fragment was introduced. The *UBI4* 5′ region was fused 7 bp upstream of the *CLN3* start codon. The resultant plasmid (YCplac111::UBI4$^{5'}$-CLN3) and its parent plasmid (YCplac111::UBI4$^{5'}$) were transformed into the wild-type haploid yeast strain JK9–3da to yield strains NB36 and NB37, respectively. Strain NB38 is JK9–3da containing the plasmid YEpURA::CLN3 (gift of K. Nasmyth), which consists of a 7-kb genomic *Bgl*II fragment containing the *CLN3* gene inserted into YEp352. For the asynchronous flow cytometry experiments, strains were grown in SD medium minus leucine to early log phase, and treated with 0.2 μg/ml rapamycin. Cell number and DNA content were analyzed hourly for 5 h following rapamycin treatment. For the synchrony experiments, NB36 and NB37 were grown to early log phase, then treated with 10 μg/ml α-factor for 2.5 h to arrest cells at start. α-Factor was removed by filtration and washing with water, followed by SD medium minus leucine, and cells were resuspended in fresh SD medium minus leucine. Samples were removed at 20-min intervals, washed, sonicated to separate cells, and assessed for emergence of buds and DNA content (flow cytometry). At maximal budding (generally 60 min after release from α-factor), the cultures were split; half received 0.2 μg/ml rapamycin, the remaining half received drug vehicle alone. Flow cytometry was performed as described above.

Assay of Starvation Sensitivity

Strain NB36 containing the *UBI4-CLN3* fusion and control strain NB37 containing the *UBI4* 5′ region without the *CLN3* open reading frame, on a *LEU2* plasmid, were grown in SD medium minus leucine for 6 days. Samples were removed daily and assessed for cell number/milliliter of culture, cell viability, and percentage of budded cells. For viability determination, 10^3 cells were plated on rich medium (YPD) in duplicate, and the number of cells able to form colonies was determined as a percentage of total number of cells plated. Replica plating to SD medium minus leucine showed that over 80% of the cells retained their respective plasmid, even after prolonged incubation.

RESULTS

Rapamycin Blocks G1 Progression

We have shown previously that rapamycin treatment causes yeast cells to arrest with an unbudded morphology (Heitman *et al.*, 1991; Kunz *et al.*, 1993). Such a phenotype, although suggestive of, is not necessarily indicative of a G1 arrest, as mutants have been isolated that are perturbed in budding but not in the onset of DNA synthesis (Adams *et al.*, 1990; Johnson and Pringle, 1990; Bender and Pringle, 1991; Cvrckova and Nasmyth, 1993). We therefore examined whether yeast cells treated with rapamycin arrest with a 1n DNA content, and are thus indeed impaired in G1 progression. An exponentially growing asynchronous culture of the haploid strain JK9–3da was treated with 0.2 μg/ml rapamycin, and at hourly intervals samples were removed for flow cytometry. As shown in Figure 1, a shift to a 1n DNA content was observed after 1 h of rapamycin treatment, and after 2–3 h, ~85% of the cells contained a 1n DNA complement (Figure 1D). The shift to 1n DNA content paralleled growth arrest; rapamycin-treated cells never completed more than one doubling, as determined by direct counting of the cells in the treated culture at the different time intervals. A control culture treated with the drug vehicle alone (10% Tween/90% ethanol) continued to grow normally, doubling in cell number every 125 min for the duration of the experiment. Thus, rapamycin causes a G1 arrest within one generation. As shown previously, TOR depletion also causes cells to arrest growth with a 1n DNA content (Helliwell *et al.*, 1994).

When the size distribution of cells was analyzed, we observed two subpopulations in the rapamycin-treated cells (Figure 1E). The major subpopulation of cells increased in size throughout the experiment, whereas the minor subpopulation of cells appeared to remain as small cells. Although the two subpopulations became more evident at later time points as the larger cells continued to increase in volume, two discrete populations could already be discerned after 2 h. The small cells most likely represent newly formed, starved daughter cells (see below) (Johnston *et al.*, 1977). The increased size of the larger cells can be accounted for by the observation that they contain an exceptionally large vacuole (Heitman *et al.*, 1991). Because an enlarged vacuole is also symptomatic of starvation (Granot and Snyder, 1991), these cells might also be starved (in G0) despite the presence of nutrients. The reason for the biphasic size distribution is unclear.

The TOR Restriction Point Is in Early G1 Before START

To determine the TOR restriction point within G1, we performed an order-of-function (reciprocal shift) analysis using a temperature-sensitive *tor* mutant and the mating pheromone α-factor (Hereford and Hartwell, 1974). This maps the TOR restriction point relative to START, the α-factor arrest point. The mutant strain (NB35) used in this experiment contained a temperature-sensitive *tor2* allele on a plasmid and chromosomal disruptions of both *TOR1* and *TOR2*. NB35

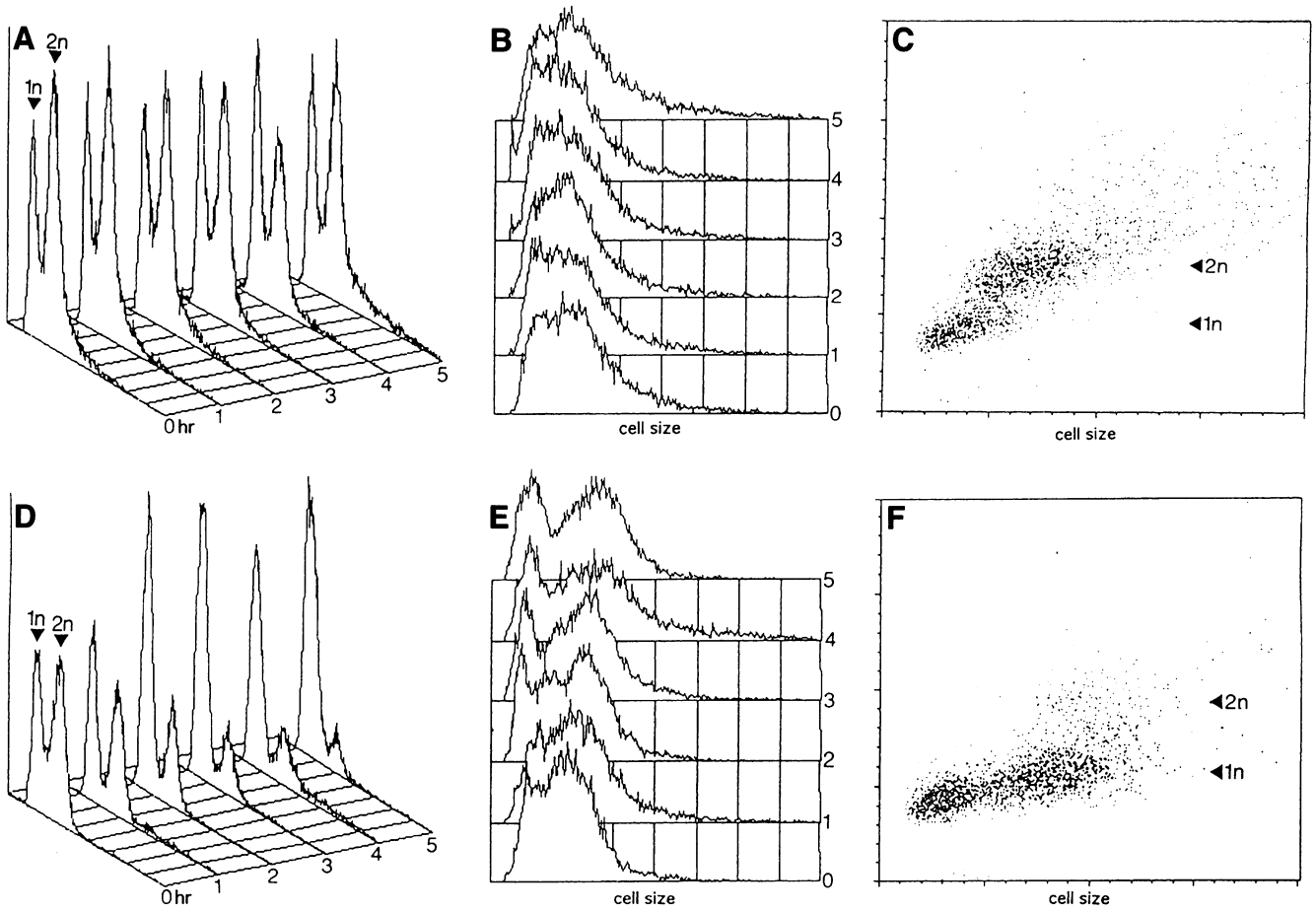


Figure 1. Rapamycin causes wild-type yeast cells (JK9–3da) to arrest with a 1n DNA content. Exponentially growing cells were treated with 0.2 μg/ml rapamycin and sampled for flow cytometry at hourly intervals up to 5 h. (A and D) DNA content, (B and E) cell size, and (C and F) a two-dimensional plot of cell size distribution (x-axis) versus DNA content (y-axis) for rapamycin-untreated (A–C) and -treated cells (D–F). The two-dimensional plot corresponds to the 5-h time point. 1n and 2n refer to DNA content.

(*tor*^{ts}) arrests growth with a 1n DNA content after shift to the nonpermissive temperature, and resumes growth upon return to the permissive temperature. The growth arrest of NB35 (*tor*^{ts}) occurs within one generation; this strain fails to complete more than one doubling after shift to the nonpermissive temperature, as determined by cell counting. Following release from a mating pheromone block and a simultaneous shift from the permissive temperature (24°C) to the nonpermissive temperature (37°C), cells synchronously entered S phase as determined by emergence of new buds (Figure 2A); cells maintained at 24°C behaved similarly. In contrast, when cells were arrested at the TOR restriction point, then released by resuspending in fresh medium at 24°C and treated with mating pheromone, they formed shmoos and did not initiate a new round of budding for the duration of the experiment (Figure 2B). Budding after shift from α-factor to the restrictive temperature was not due to a slow inactivation of temperature-sensitive TOR. First, NB35 (*tor*^{ts}) arrests within one generation. Second, shifting cells to the nonpermissive temperature 1.5 h before release from the α-factor block did not prevent budding (Figure 2C). Third, wild-type cells released from an α-factor block into medium containing rapamycin also resumed budding (Figure 2D). The results of a double block experiment performed with α-factor and rapamycin (see MATERIALS AND METHODS) were also consistent with a TOR restriction point in early G1; rapamycin prevented nocodazole-synchronized cells from forming shmoos in response to α-factor (our unpublished results). Thus, the TOR restriction point is in early G1 before START.

As further evidence that loss of TOR function causes an early G1 arrest, we observed that rapamycin-treated cells lack START-specific transcripts encoding the G1 cyclins (Figure 3) (see below), and that providing *CLN2* under control of the rapamycin-unresponsive, constitutive *Schizosaccharomyces*

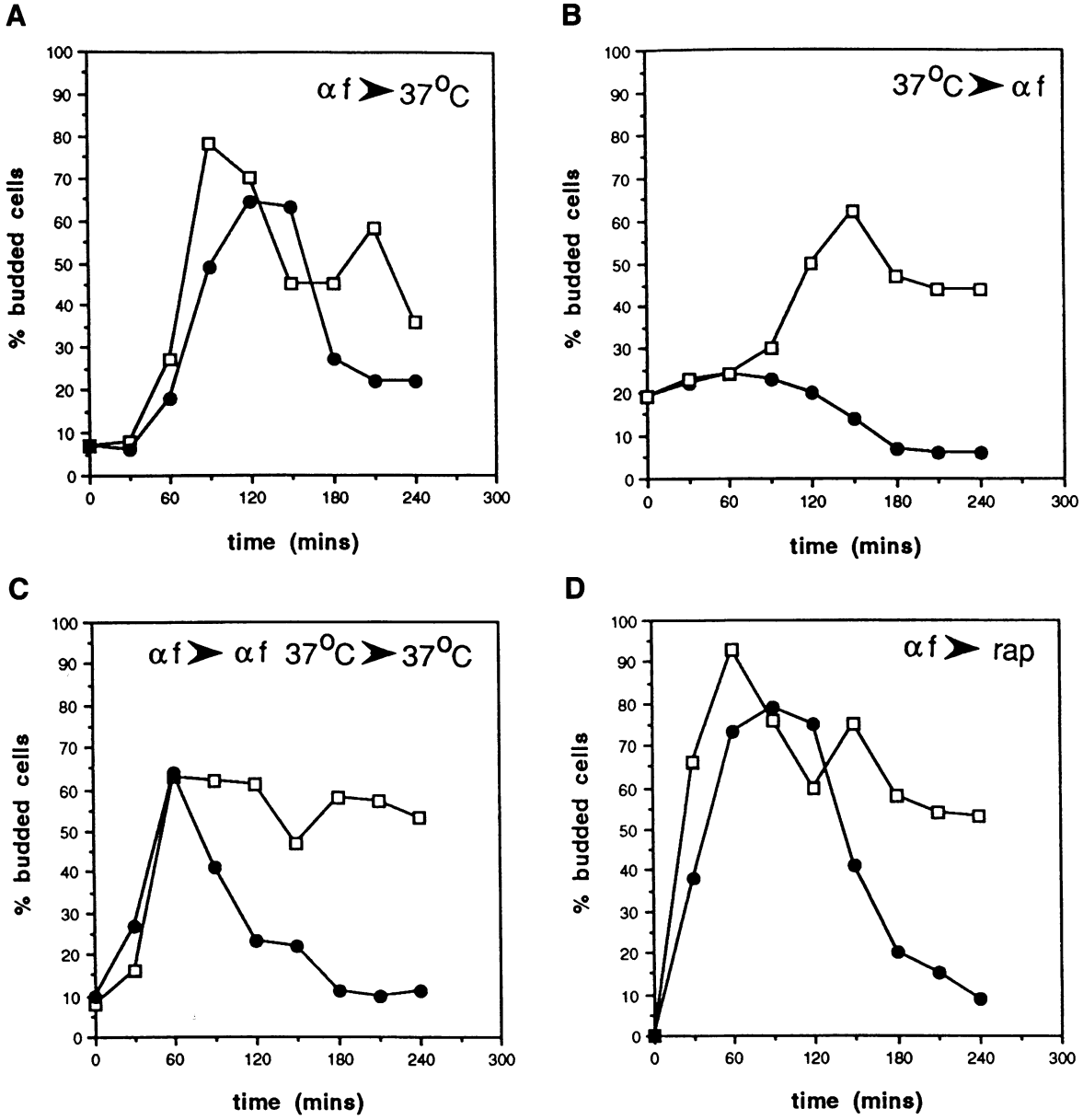


Figure 2. TOR depletion arrests cells in early G1 before START. (A–C) Order-of-function determination by an α-factor (αf) and *tor*^{ts} reciprocal shift. The percentage of budded cells was monitored at the indicated time points after release from the first block. (A) Strain NB35 (*tor*^{ts}) was arrested by pretreatment with α-factor at the permissive temperature, washed, and resuspended in fresh medium without α-factor at 24°C (open squares) or 37°C (closed circles). (B) Strain NB35 (*tor*^{ts}) was arrested by preincubation for 5 h at the nonpermissive temperature (37°C), washed, and resuspended in fresh medium at the permissive temperature (24°C) containing vehicle alone (open squares) or α-factor (closed circles). (C) Strain NB35 (*tor*^{ts}) was treated as in panel A with the modification that cells were shifted to the nonpermissive temperature 1.5 h before release from the α-factor block. (D) Order-of-function determination by an α-factor to rapamycin shift. Wild-type strain JK9–3da was arrested at START by α-factor treatment, washed, and resuspended in medium containing vehicle alone (open squares) or 0.2 μg/ml rapamycin (closed circles). The percentage of budded cells was determined at the indicated times after release from α-factor.

pombe ADH promoter (Nasmyth and Dirick, 1991) does not abrogate the rapamycin-induced cell cycle arrest (our unpublished results). Thus, TOR is not directly (or solely) required for *CLN* gene transcription, and the loss of START-specific transcripts is a downstream effect rather than the direct cause of the cell cycle arrest. A constitutively expressed *CLN2* transcript does not suppress the rapamycin-induced cell cycle arrest presumably because it is not translated (see below).

The effects of rapamycin treatment on START-specific transcripts were as follows. The mRNAs for *CLN1*

and *CLN2* (Figure 3 and our unpublished results for *CLN2*) were no longer detectable after 2 h of rapamycin treatment. Surprisingly, the normally constitutively expressed *CLN3* transcript was also reduced with similar kinetics as seen for *CLN1* and *CLN2*, but was not completely eliminated. As determined by densitometry of appropriately exposed autoradiographs and normalization to *ACT1* transcript levels, the *CLN3* mRNA level was maximally reduced by ~60%. The mRNAs for the three additional genes, *HCS26*, *ORFD*, and *CLB5*, which bear limited homology to the *CLN* genes and are also expressed only in late G1 also disappeared upon rapamycin treatment, with kinetics identical to those seen for the *CLN1* and *CLN2* transcripts (Figure 3 for *HCS26* and *ORFD*).

Expression of the *CLN1*, *CLN2*, and *HCS26* genes (and possibly *ORFD*) is under control of the transcription factor SBF, which is composed of the DNA binding moiety SWI4 and its regulatory subunit SWI6 (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). We therefore assessed the levels of *SWI4* and *SWI6* transcripts in rapamycin-treated cells. Normally, the mRNA for SWI6 is constitutively expressed whereas the mRNA for SWI4 oscillates, peaking in late G1 and falling to a low but detectable basal level elsewhere in the cell cycle (Breeden and Mikesell, 1991). Like the *CLN3* transcript, the mRNA for SWI6 was depleted by ~60% (Figure 3). The transcript for SWI4 fell to basal levels 2 h after rapamycin treatment, thus behaving like other START-specific mRNAs.

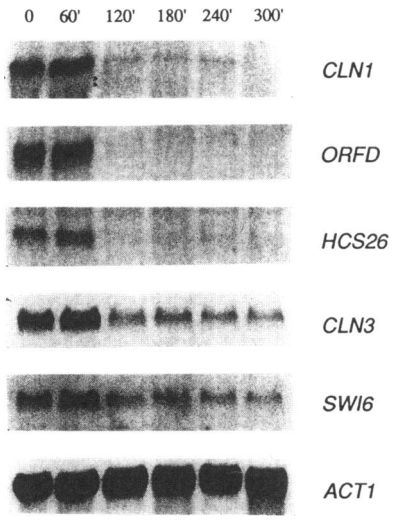


Figure 3. START-specific transcripts are depleted upon rapamycin treatment. Northern blot analysis of RNA isolated from cells (JK9–3da) treated with 0.2 μg/ml rapamycin for 0, 1, 2, 3, 4, and 5 h (indicated in minutes). The mRNAs for *CLN1*, *ORFD*, and *HCS26* are abolished, and mRNAs for *CLN3* and *SWI6* are reduced by ~60% relative to *ACT1* levels. *ACT1* encodes actin and is a control for a message that is not START specific. The level of *ACT1* message is not affected by rapamycin. See text for additional information.

These observations are not due to a global repression of transcription as the transcripts for actin (*ACT1*) and *CDC28* and also the previously identified targets of rapamycin *TOR1* and *TOR2* were not depleted throughout the time course of these experiments (Figure 3 and our unpublished results). Furthermore, some transcripts are actually induced upon rapamycin treatment (see below). As mentioned above, the absence of START-specific transcripts upon rapamycin treatment is presumably an indirect consequence of a cell cycle arrest before START (Hubler *et al.*, 1993). The reduction in the normally constitutive messages could reflect the inherent instability of untranslated (see below) mRNAs.

TOR Is Required for Translation Initiation

Because rapamycin blocks activation of protein synthesis in mammalian cells (Jefferies *et al.*, 1994; Terada *et al.*, 1994) and because inhibition of protein synthesis in yeast causes an early G1 arrest (Hartwell and Unger, 1977; Pringle and Hartwell, 1981; Brenner *et al.*, 1988), we investigated whether rapamycin blocks protein synthesis in yeast by assaying incorporation of [^{35}S]methionine at intervals after addition of rapamycin. We observed an early decrease in incorporation upon rapamycin treatment (Figure 4A). Protein synthesis fell to a low (~10% of normal levels) but detectable level after 120 min, and remained at this low level throughout the course of the experiment. The low level of protein synthesis was greater than that observed in cells treated with cycloheximide (100 μg/ml), which reduced protein synthesis to undetectable levels. Up to 100-fold higher concentrations of rapamycin did not have a more severe effect on incorporation. Protein synthesis was not affected in a rapamycin-resistant *TOR1–1* (JH11–1c) or *TOR2–1* (JH12–17b) mutant, as assayed by [^{35}S]methionine incorporation in the presence of rapamycin. Thus, rapamycin is an effective inhibitor of protein synthesis acting through TOR.

To confirm that TOR is required for protein synthesis, as suggested by the above observation, we examined the effect of TOR depletion on protein synthesis. The *tor*^{ts} strain NB35 was shifted to the nonpermissive temperature and levels of protein synthesis were determined at time intervals after the temperature shift. At the nonpermissive temperature, we observed a progressive decrease in the levels of [^{35}S]methionine incorporation (Figure 4B). Incorporation levels fell to a minimum of ~10% after 6 h of incubation at the nonpermissive temperature. Levels of incorporation in NB35 (*tor*^{ts}) at the permissive temperature were less than those in wild type, indicating that there is a protein synthesis defect in this mutant even at the permissive temperature. Thus, TOR is required for protein synthesis. Furthermore, because an inhibi-

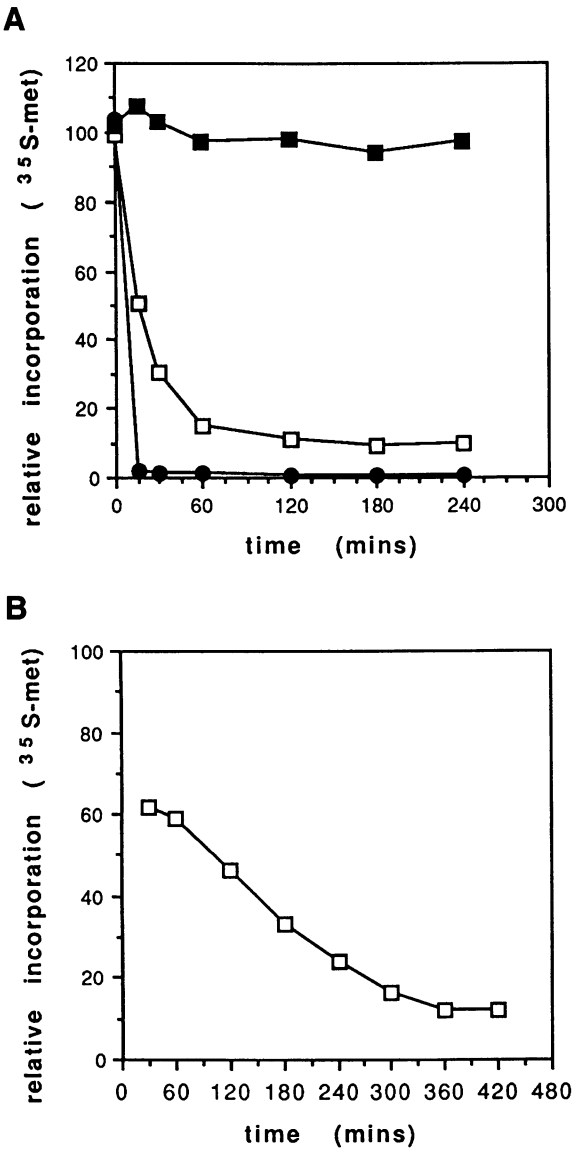


Figure 4. Rapamycin treatment inhibits protein synthesis. (A) Cells were assessed for incorporation of [^{35}S]methionine by labeling for 7 min at intervals (0, 15, 30, 60, 120, 180, and 240 min) after addition of 0.2 μg/ml rapamycin. Cells treated were wild-type JK9–3da (open squares) and rapamycin-resistant *TOR1–1* mutant JH11–1c (closed squares). Also plotted is JK9–3da treated with 100 μg/ml cycloheximide (closed circles). Incorporation (relative incorporation) is plotted as a percentage of the control, wild-type strain JK9–3da treated with drug vehicle alone. (B) Inhibition of protein synthesis upon TOR depletion. Strain NB35 (*tor*^{ts}) was incubated at the restrictive temperature, and samples were removed at the indicated time intervals for determination of [^{35}S]methionine incorporation. Values are plotted as a percentage of [^{35}S]methionine incorporation in NB17–3d at the restrictive temperature. An early time point is not included because a reliable value could not be obtained for either the temperature-sensitive mutant NB35 or NB17–3d immediately after shift to the nonpermissive temperature. Shown (A and B) are representative curves of three or more independent experiments.

tion of protein synthesis causes an early G1 arrest (Hartwell and Unger, 1977; Pringle and Hartwell, 1981; Brenner *et al.*, 1988), the protein synthesis defect may be the cause of the cell cycle arrest; the relatively slow inhibition of incorporation in NB35 (*tor*^{ts}) at the nonpermissive temperature, compared with rapamycin-treated cells at the permissive temperature, is not necessarily inconsistent with the first cycle arrest of NB35 (*tor*^{ts}) because this strain has a translation defect even at the permissive temperature and because cells have a longer cell cycle at the higher temperature.

To determine whether the inhibition of protein synthesis was at the level of initiation or elongation, polysome profiles of wild-type cells treated with rapamycin for 1 and 2 h were analyzed. This experiment was performed in the absence of the translation elongation inhibitor cycloheximide so that a block in elongation, if imposed, could be observed. Such a block is characterized by an accumulation of polysomes. No polysomes were present in either extract, only a single peak corresponding to 80S monosomes and ribosomes (our unpublished results). Rapamycin does not, therefore, cause a translation elongation block; however, a mild defect in the rate of elongation that is not sufficiently stringent to prevent ribosome "run-off" during the time needed to harvest, wash, and lyse cells in preparation for sucrose gradients would not be detected. To investigate whether rapamycin causes a block in translation initiation, wild-type cells were treated with drug vehicle alone or with rapamycin for 1 and 2 h followed by a 10-min treatment with cycloheximide to prevent run-off of any polysomes present (Figure 5, A and B; our unpublished result for 1-h timepoint). Rapamycin treatment caused a progressive decay of polysomes with a coincident increase in the 80S peak, indicating an initiation block. The apparent discrepancy between the observed inhibition of [^{35}S]methionine incorporation (~90%) and the inhibition of polysomes (~60%) after 2 h of rapamycin treatment may reflect a difference in the sensitivities of the two assays or a mild elongation defect in addition to a block in initiation.

We next examined the polysome profiles of TOR-depleted cells using the *tor*^{ts} strain NB35. Again, a severe reduction in the number of polysomes and a coincident increase in the 80S peak were evident after incubation for 5 h at the nonpermissive temperature (Figure 5C). A similar but less pronounced effect was observed after 3 h at the nonpermissive temperature. Thus, TOR is required for translation initiation.

Loss of TOR Causes a Starvation Response, but TOR Is Not Part of the RAS/cAMP Pathway

Starved yeast cells exit the cell cycle (stop dividing) and enter G0. Cells entering G0 are characterized by

several distinct properties (Werner-Washburne *et al.*, 1993) including 1n DNA content, failure to reach START (Pringle and Hartwell, 1981), a reduction in protein synthesis to ~10% of normal levels, down-regulation of *CLN3* message (Hubler *et al.*, 1993), and enlargement of the vacuole (Granot and Snyder, 1991). As described above, rapamycin-treated or TOR-depleted cells display all these characteristics. Additionally, rapamycin-treated or TOR-depleted cells are still alive (metabolically active) despite the observed reduction in protein synthesis; rapamycin-treated cells exclude the vital dye phloxin B even 24 h after treatment, and all temperature-sensitive *tor2* alleles isolated to date are reversible (Barbet and Hall, unpublished data). This led us to consider that rapamycin might be causing a starvation response despite the presence of nutrients, and inducing cells to enter G0. To test this, we examined by Northern analysis the effect of rapamycin on the transcription of genes whose mRNA levels are known to change upon entry into G0. The heat shock genes *SSA3* and *HSP26* and the ubiquitin gene *UBI4* are transcriptionally induced upon entry into G0 (Werner-Washburne *et al.*, 1993). The catalase T gene *CTT1* is also transcriptionally induced upon entry into G0, with enzymatic activity peaking and then declining 3 h after cells enter stationary phase (Werner-Washburne *et al.*, 1993). In contrast, the mRNA level of the heat shock genes *SSA1* and *SSA2* (*SSA1/2*) fluctuates in different ways depending on the starvation regimen but can remain largely unchanged, and transcription of the cold-inducible "heat shock" genes *SSB1* and *SSB2* (*SSB1/2*) is severely repressed upon entry into G0 (Werner-Washburne *et al.*, 1993). As shown in Figure 6A, we observed these same changes in transcription upon rapamycin treatment. The mRNAs for *SSA3*, *HSP26*, and *UBI4* were induced upon rapamycin treatment; maximal induction occurred 2 h after rapamycin addition for *SSA3* and *HSP26*, and after 30 min for *UBI4*. The *CTT1* transcript was also induced upon rapamycin treatment, and transcript levels remained high for 2 h before falling. In contrast, the *SSB1/2* transcripts decreased to almost undetectable levels within 1 h of treatment. The level of *SSA1/2* transcripts fluctuated but remained largely unchanged. Thus, it appears that rapamycin causes a starvation response and induces entry into G0.

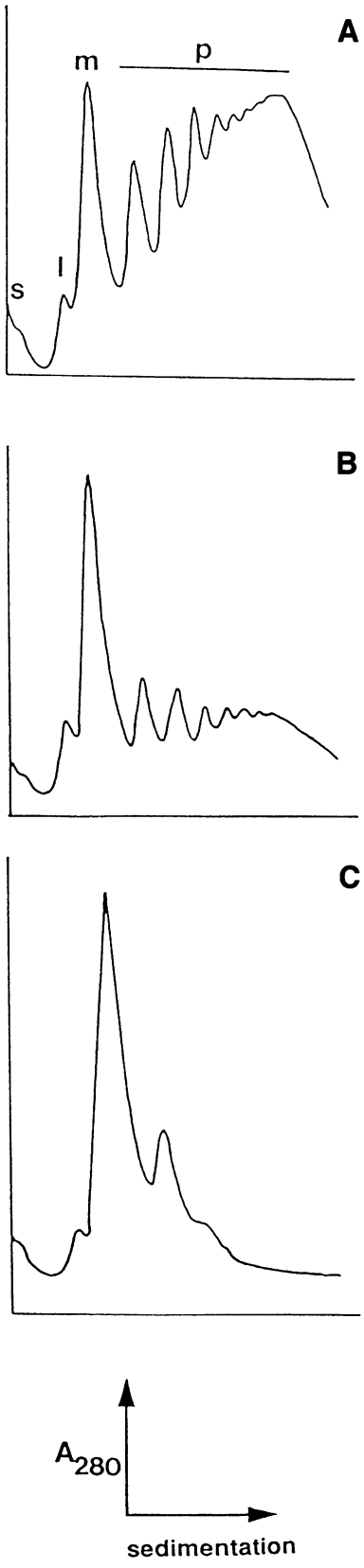
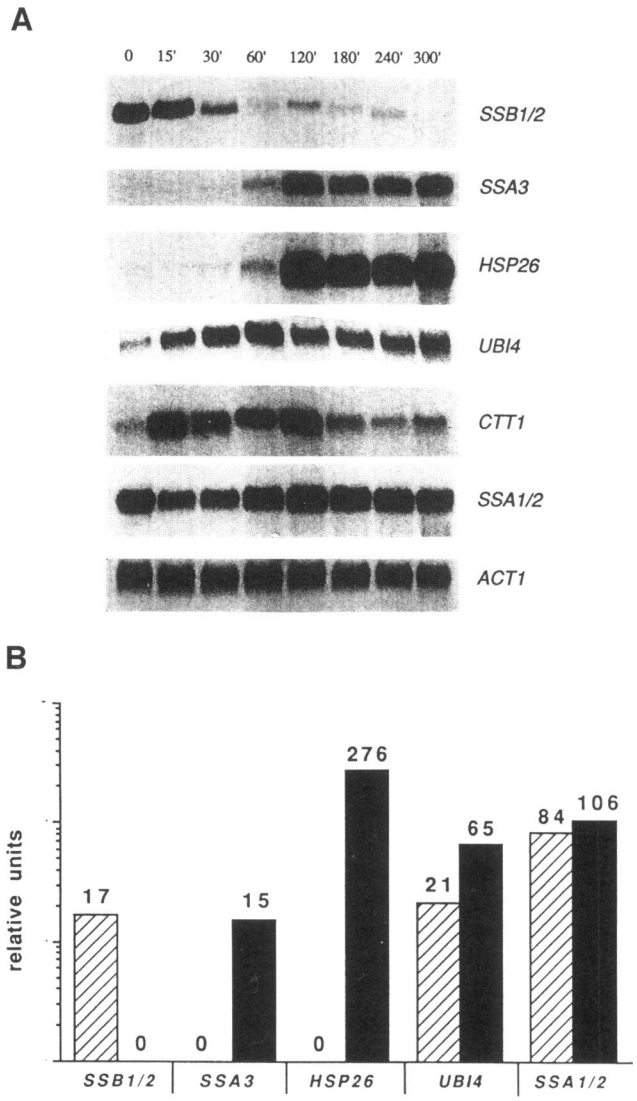


Figure 5. Rapamycin treatment or TOR depletion blocks translation initiation. (A and B) Polysome profiles of wild-type cells (JK9–3da) treated with (A) vehicle alone, and (B) 0.2 μg/ml rapamycin for 2 h. (C) Polysome profile of the *tor*^{ts} strain NB35 after 5 h at the nonpermissive temperature (37°C). In all the above cases, cycloheximide was added 10 min before harvest, to prevent "run off." Wild-type strain JK9–3da grown at the nonpermissive temperature is slightly stimulated for polysome accumulation. The positions of 40S ribosomal subunits (s), 60S ribosomal subunits (l), 80S monosomes (m), and polysomal ribosomes (p) are indicated.



We next examined whether TOR depletion elicits the same starvation-induced changes in transcript levels. We could not utilize the *tor*^{ts} strain for these experiments, because many of the same changes in transcript levels occur normally at high temperature (the nonpermissive temperature of our *tor*^{ts} mutant) independently of starvation. Therefore, we used a strain containing *TOR2* under control of the regulatable *GAL1* promoter and chromosomal disruptions of both *TOR1* and *TOR2* (JK350–21a) to deplete the cells of TOR (Kunz *et al.*, 1993; Helliwell *et al.*, 1994). After shifting from galactose- (SGal/Gly) to glucose-containing (SD) medium (TOR-depletion conditions), we observed changes in the pattern of transcription similar to those seen when wild-type cells are treated with rapamycin (Figure 6B). Thus, TOR depletion also induces a starvation response.

Additional indicators of stationary phase are the accumulation of the storage carbohydrate glycogen and acquisition of thermotolerance. We examined whether cells treated with rapamycin accumulate glycogen. Cultures were treated for 5 h with rapamycin, harvested by filtration at hourly intervals, and stained for glycogen using iodine vapor, which stains glycogen-containing cells dark brown (Chester, 1968). As shown in Figure 6C for the 5-h time point, cells treated with rapamycin did indeed stain darkly when exposed to iodine. Accumulation of glycogen was weakly detectable after 1 h of treatment. Also confirming that loss of TOR function induces a starvation response, we observed that cells depleted for TOR exhibit increased resistance to the killing effects of high temperatures when compared with wild-type cells (our unpublished results).

The RAS/cAMP signal transduction pathway acts in early G1 (before the mating pheromone arrest point) and may be involved in the controlled entry into G0 (Broach, 1991; Thevelein, 1994). To investigate

Figure 6. Rapamycin treatment or TOR depletion induces a starvation response. (A) RNA was isolated from cells (JK9–3da) treated with 0.2 μg/ml rapamycin for 0, 15, 30, 60, 120, 180, 240, and 300 min, and probed by Northern analysis with the indicated genes (see MATERIALS AND METHODS). *SSA1/2* refers to *SSA1* and *SSA2*. *SSB1/2* refers to *SSB1* and *SSB2*. The observed changes in transcript levels are characteristic of cells entering G0. (B) Histogram showing level changes for the indicated transcripts upon depletion of TOR by galactose to glucose shift. Conditions of the galactose to glucose shift were as described (Helliwell *et al.*, 1994). Hatched bars correspond to a wild-type (JK9–3da) strain; solid bars correspond to a TOR-depleted strain (JK350–21a). Transcript levels were normalized to *ACT1* mRNA levels. Transcript level values in relative units are given above each bar. A value of 0 indicates an undetectable mRNA level. (C) Rapamycin treatment causes accumulation of glycogen. (Top left) Rapamycin-resistant strain JH12–17b treated with drug vehicle alone. (Top right) JH12–17b treated for 5 h with 0.2 μg/ml rapamycin. (Bottom left) Wild-type strain JK9–3da treated with drug vehicle alone. (Bottom right) JK9–3da treated for 5 h with 0.2 μg/ml rapamycin. Filters were exposed to iodine vapor for 1 min to stain for glycogen.

whether loss of TOR function induces entry into G0 by inhibiting the RAS/cAMP cascade, we constitutively activated this pathway, and then tested for abrogation of the rapamycin-induced cell cycle arrest. Two methods were used to constitutively activate the pathway. First, we disrupted the *BCY1* gene (Toda *et al.*, 1987). A *BCY1* disruption activates the RAS/cAMP pathway by eliminating the negative regulatory subunit of the cAMP-dependent protein kinase A (Cannon and Tatchell, 1987; Toda *et al.*, 1987). Second, we introduced the dominant, activated *RAS2* allele $RAS2^{val19}$. The $RAS2^{val19}$ mutation hyperactivates the RAS/cAMP pathway by maintaining RAS2 in its active, GTP-bound state (Kataoka *et al.*, 1984). Both *bcy1* (NB30) and $RAS2^{val19}$ (NB34) cells were as sensitive as wild-type cells to rapamycin, based upon growth arrest in the presence of drug. Flow cytometry on these strains indicated that greater than 85% of the cells arrested with a 1n DNA content after 3 h of rapamycin treatment, as observed with wild-type cells (see Figure 1 for wild-type cells). Rapamycin-treated *bcy1* and $RAS2^{val19}$ cells also accumulated glycogen, as determined by iodine staining. Therefore, activation of the RAS/cAMP pathway does not abrogate the rapamycin-induced cell cycle arrest, indicating that TOR is not part of the RAS/cAMP pathway.

Our data do not rule out the possibility that TOR lies in the RAS/cAMP pathway downstream of BCY1, but we consider this very unlikely. First, subcellular localization studies (Kunz, Stevenson, Schneider, and Hall, unpublished data) and their homology to lipid kinases indicate that the TORs are membrane-associated proteins, whereas BCY1 is a membrane-distal component of the RAS/cAMP pathway. Second, diploid cells lacking TOR function arrest in G1 (2n DNA content) but do not sporulate, whereas diploids compromised in the RAS/cAMP pathway do sporulate. Third, activation of p70 S6 kinase, a presumed downstream component of TOR in mammalian cells, is independent of $p21^{ras}$ (Downward, 1994; Ming *et al.*, 1994). Fourth, there is no example of, or need for, a lipid kinase in a signaling pathway that utilizes cAMP as a second messenger; the lipid kinases mediate production of the fundamentally different, phosphatidylinositol-derived second messengers. Thus, TOR1 and TOR2 appear to define a novel nutrient-related process mediating progression through early G1. This would be in agreement with the observations of Cameron *et al.* (1988), who described mutants that express low-level, constitutive cAMP-dependent protein kinase A activity but that still respond appropriately to nutrient conditions, even in the absence of essential upstream components of the RAS/cAMP pathway.

Expression of CLN3 under Altered Translational Control Confers TOR-independent G1 Progression

The finding that loss of TOR function causes an early reduction in protein synthesis and a G1 arrest within one generation suggested that TOR might be controlling translation of an unstable protein(s) required for G1 progression. Good candidates for such proteins were the G1 cyclins, as these proteins are unstable and limiting for G1 progression (Cross, 1988; Nash *et al.*, 1988; Hubler *et al.*, 1993; Tyers *et al.*, 1993). To test whether cells lacking TOR function arrest in early G1 (G0) because they do not synthesize G1 cyclins, we devised a situation in which one of these, CLN3, would be synthesized upon rapamycin treatment, and asked whether this would be sufficient to drive rapamycin-treated cells through G1. CLN3 was chosen because the transcript for this cyclin is normally present under conditions of rapamycin treatment (Figure 3). We fused the *CLN3* open reading frame to the 5′ region (untranslated leader and promoter) of the *UBI4* gene. The *UBI4* 5′ region was chosen because it is both transcriptionally and translationally active in G0 and would therefore express CLN3 upon rapamycin treatment (Finley *et al.*, 1987; Brenner *et al.*, 1988; Werner-Washburne *et al.*, 1993) (Figure 6A). We then examined whether the *UBI4-CLN3* fusion suppresses the rapamycin-induced cell cycle arrest.

An asynchronously growing wild-type yeast strain containing the *UBI4-CLN3* fusion on a centromeric plasmid (NB36) was treated with rapamycin, and at hourly intervals the DNA content of the cells was analyzed by flow cytometry. Like a control strain (NB37) containing a plasmid-borne *UBI4* 5′ region without the *CLN3* open reading frame, NB36 cells arrested growth after approximately 2 h of rapamycin treatment. This was expected because rapamycin causes a general inhibition of protein synthesis (Figure 4), and TOR has an essential non-cell cycle function in addition to its essential role in G1 (Kunz *et al.*, 1993). Analysis of DNA content of the arrested cells, however, indicated that NB36 (*UBI4-CLN3*) arrested throughout the cell cycle, whereas the control strain arrested in G1 (Figure 7). Thus, cells containing the *UBI4-CLN3* fusion no longer arrest in G1 upon rapamycin treatment.

Northern analysis of the strain (NB36) containing the *UBI4-CLN3* fusion indicated that it produces approximately 20-fold more *CLN3* mRNA upon rapamycin treatment than an isogenic strain lacking the fusion. To determine whether the suppression of the cell cycle arrest in strain NB36 was due to altered control of CLN3 translation or merely to the increased dosage of the *CLN3* transcript, we examined whether cells containing the wild-type *CLN3* gene on a high-copy-number plasmid (NB38) also arrested outside of G1 upon rapamycin treatment. After 2 h of treatment,

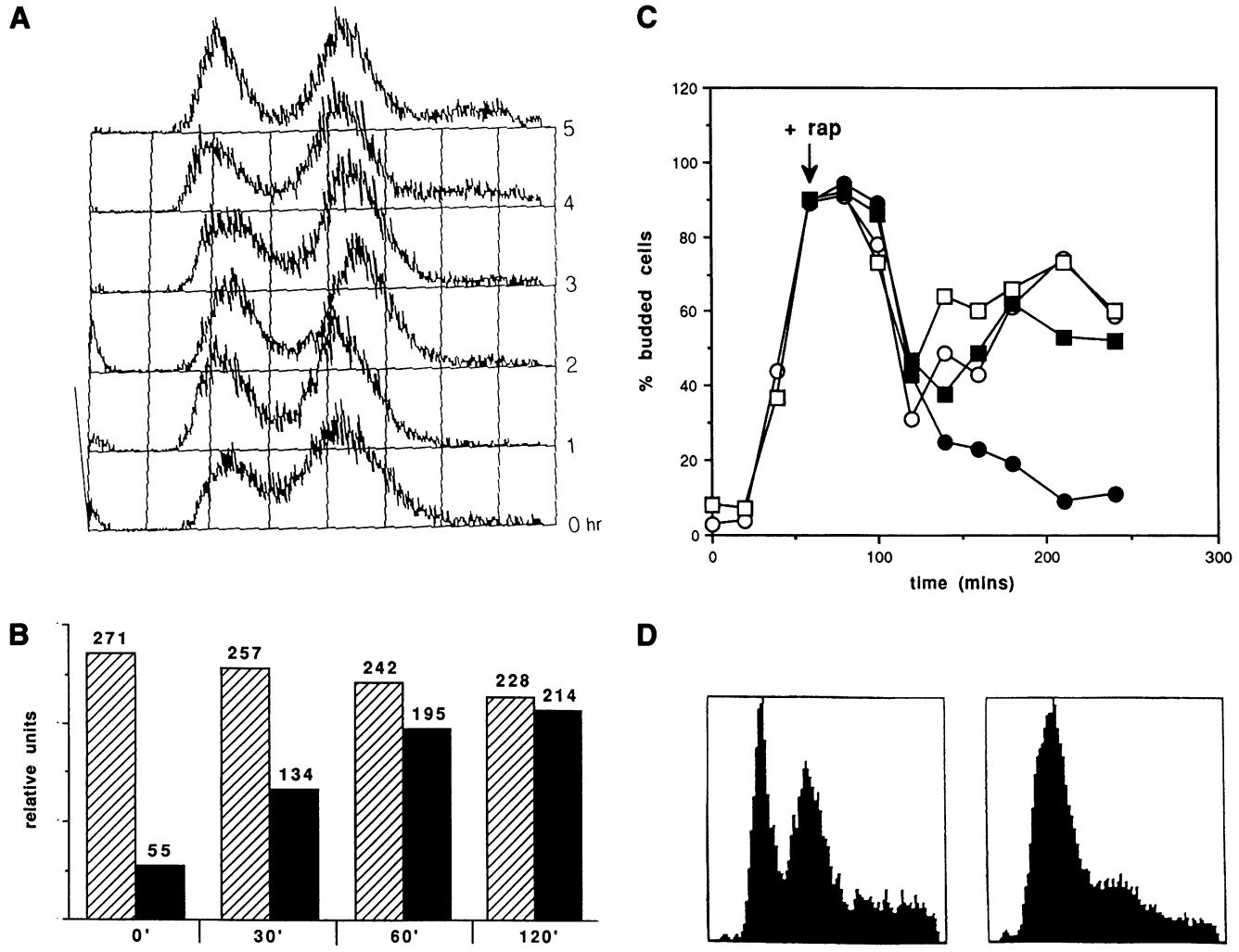


Figure 7. Altered translational control of CLN3 suppresses the cell cycle–specific arrest of rapamycin-treated cells. (A) Exponentially growing cells containing the *UBI4-CLN3* fusion (NB36) were treated with 0.2 μg/ml rapamycin and sampled for flow cytometry at hourly intervals up to 5 h. Rapamycin-treated NB36 arrested throughout the cell cycle, as indicated by a roughly even distribution of cells with a 1n and 2n DNA content. Results of flow cytometry on control strain NB37 treated with rapamycin were indistinguishable from the results in Figure 1D. (B) Northern analysis of strains treated with rapamycin and assessed for levels of *CLN3* transcript. Shown are the levels of *CLN3* transcript in strain NB38 (hatched bars) containing the wild-type *CLN3* gene in a high copy number plasmid and strain NB36 (solid bars) containing the *UBI4-CLN3* fusion, at the indicated times (in minutes) following rapamycin treatment. All values were normalized to the levels of actin transcript. At 120 min following treatment, cells had arrested growth. (C and D) A *UBI4-CLN3* strain treated with rapamycin is able to traverse G1. (C) Percentage of budded cells of rapamycin-treated (closed squares) or -untreated (open squares) NB36 (*UBI4-CLN3*) compared with rapamycin-treated (closed circles) or -untreated (open circles) control strain NB37. Percentage of budded cells was determined at 20-min intervals following release from the α-factor block at START. Rapamycin (rap) was added 60 min following release from α-factor. (D) Flow cytometry of NB36 (*UBI4-CLN3*) and NB37 control cells released from an α-factor block at time 0 and treated with rapamycin 60 min following release from α-factor. Shown is the DNA content of the rapamycin-treated cells at the end of the experiment (240 min). The rapamycin-treated cells arrested growth approximately 150 min after rapamycin addition. The left panel shows the DNA content for strain NB36, the right panel shows the DNA content for NB37 (G1 arrest). DNA content of untreated cells at 240 min was indistinguishable from that shown in the left panel. Data shown is representative of three independently performed experiments.

NB38 cells arrested growth, with ~85% of cells containing a 1n DNA content. Northern analysis of the rapamycin-treated NB36 (*UBI4-CLN3*) and NB38 (high copy *CLN3*) cells indicated that the level of CLN3 transcripts in NB38 was greater than that in NB36 (Figure 7B). In addition, high level overexpression of *CLN3* from the inducible *GAL1* promoter was also unable to overcome a rapamycin-induced G1 arrest, and plasmid-borne *UBI4-CLN3* still caused a random arrest despite disruption of the chromosomal copy of

CLN3 (our unpublished results). Furthermore, an integrated copy of the *CLN3–1* allele (strain NB33), which bears a mutation that stabilizes the CLN3 protein but does not otherwise affect its cyclin function (Cross, 1988; Nash *et al.*, 1988), had the same effect as *UBI4-CLN3* in causing a random arrest upon rapamycin treatment. This confirms that the *UBI4-CLN3* fusion does not promote G1 progression simply because of an elevated level of *CLN3* transcripts.

To determine more directly whether rapamycin-treated cells containing the *UBI4-CLN3* fusion are able to traverse the G1 phase of the cell cycle, we examined the effect of rapamycin on synchronized cells. Strain NB36 (*UBI4-CLN3*) and the control strain NB37 were synchronized at START by addition of α-factor. Following release from the pheromone block, the cultures were split into two and rapamycin was added to one half, the remaining halves receiving drug vehicle alone. As shown in Figure 7C, NB37 control cells treated with rapamycin entered G1 and arrested as unbudded cells. Rapamycin-treated NB36 (*UBI4-CLN3*), however, entered G1 but then began to produce new buds before arresting growth, indicating that cells were traversing G1 and beginning a new cycle. Analysis of the arrested cells by flow cytometry confirmed that the NB36 (*UBI4-CLN3*) cells had traversed G1 whereas the NB37 control cells had not (Figure 7D). Thus, *UBI4* leader-dependent expression of CLN3 causes rapamycin-treated cells to traverse G1.

Expression of UBI4-CLN3 Confers Starvation Sensitivity

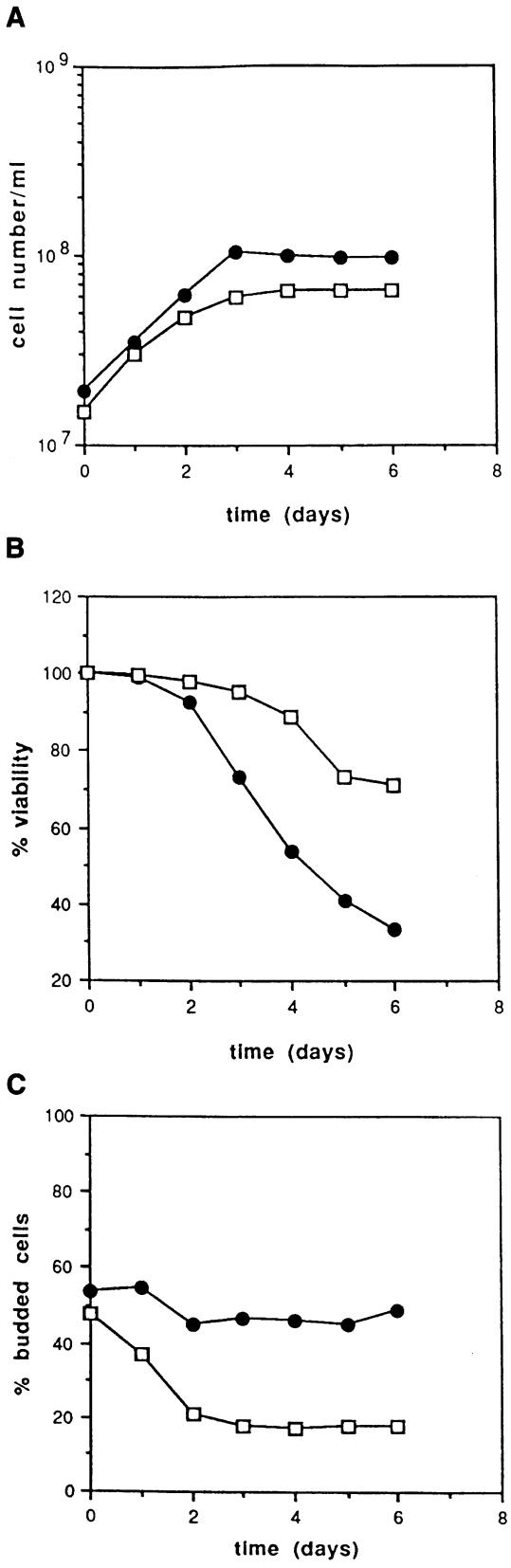
The finding that TOR may normally modulate synthesis of CLN3 (among other proteins) as part of a starvation response suggested that cells containing the *UBI4-CLN3* fusion might be sensitive to starvation. To test this suggestion, *UBI4-CLN3* strain NB36 and control strain NB37 were grown to stationary phase (starved) and samples were removed daily for assessment of cell viability and the percentage of budded cells. As NB36 (*UBI4-CLN3*) cells entered stationary phase (cell number no longer increased) (Figure 8A), their ability to form colonies on rich medium rapidly decreased (Figure 8B). In contrast, starved NB37 control cells retained high viability for the duration of the experiment. The starvation sensitivity of NB36 (*UBI4-CLN3*) was most likely due to this strain's inability to arrest in G1 (G0), as suggested by the observations that it stopped dividing at a higher cell density (~1.5-fold) than the control strain (Figure 8A) and with a high percentage of budded cells (Figure 8C). Strain NB38 containing the wild-type *CLN3* gene in high dosage behaved in this experiment like control strain NB37. These findings support the involvement of TOR in nutrient sensing, and also confirm that modulating the level of translation is part of the regulated entry into stationary phase (G0).

DISCUSSION

We have shown that loss of TOR function (rapamycin treatment or TOR depletion) causes yeast cells to arrest in early G1 and to exhibit, by all criteria examined, characteristics of starved cells entering stationary phase, or G0. We have also demonstrated that loss of TOR function causes a general inhibition of translation initiation. Providing the transcript for the G1 cyclin CLN3 under the translational control of the *UBI4* 5′ region suppresses the rapamycin-induced G1 arrest and confers starvation sensitivity. These results suggest the following model for the role of TOR in cell cycle control (Figure 9). In response to nutrient availability, TOR stimulates general translation initiation, including translation of G1-regulatory transcripts such as those for CLN3 and other G1 cyclins. This then drives cells through G1 and into S phase. In the converse situation, the absence of nutrients causes inactivation of TOR, which leads to loss of translation and a subsequent early G1 arrest and entry into G0. It is important to emphasize that TOR is required for general translation and that the role of TOR in cell cycle control, as proposed here, is just part of a greater role in general growth control. We would also like to stress that, although it accounts for all our data, the model is largely speculative and is intended only as a framework to bring together our and other findings.

Several lines of evidence suggest that TOR is part of a signaling pathway. First, the TORs are homologous to PI kinases, enzymes implicated in signaling. Second, because loss of TOR rapidly causes a starvation response, TOR is likely involved in sensing and relaying the availability of nutrients. Indeed, constitutively activating the proposed pathway by providing CLN3 independently of upstream components (*CLN3* under the translational control of the *UBI4* untranslated leader) causes starvation sensitivity. Third, the mammalian counterpart of TOR (FRAP/RAFT1/RAPT1/mTOR) appears to mediate an intermediate step in a defined, rapamycin-sensitive signal transduction pathway required for cell proliferation (Brown *et al.*, 1994; Chiu *et al.*, 1994; Downward, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995). The putative TOR pathway is novel because it acts in early G1, and TOR is not part of the RAS/cAMP pathway.

The observed inhibition of translation initiation is likely a direct consequence of loss of TOR function and the cause (rather than an effect) of the cell cycle arrest, for the following reasons. First, the reduction in translation is the earliest effect observed upon loss of TOR function. Second, a specific block in translation initiation, either by mutation of an initiation factor or by treatment with a low concentration of cyclo-



heximide, causes yeast cells to arrest in early G1 (Hartwell and Unger, 1977; Johnston *et al.*, 1977; Pringle and Hartwell, 1981; Hanic-Joyce *et al.*, 1987; Brenner *et al.*, 1988; Hubler *et al.*, 1993; Barnes *et al.*, 1995). Third, and most important, allowing translation initiation of an appropriate, cell cycle–controlling transcript is sufficient to suppress the rapamycin-induced G1 arrest. Fourth, TOR in mammalian cells probably activates translation initiation and G1 progression in response to mitogens (Downward, 1994; see INTRODUCTION). Thus, the TOR pathway in yeast appears to control translation initiation and, thereby, early G1 progression.

The observation that phosphorylation of the yeast equivalent of S6 (S10) is not important for growth (Zinker and Warner, 1976; Kruse *et al.*, 1985; Johnson and Warner, 1987) suggests that TOR is not regulating translation initiation in yeast through S6 (see INTRODUCTION). One alternative possibility is that the TOR pathway controls translation initiation through the initiation factor eIF-4E (or an associated subunit). eIF-4E is the cap-binding subunit of the eIF-4F complex, which also contains eIF-4A, an RNA helicase, and eIF-4γ, a protein of unknown function (Rhoads, 1988; Lanker *et al.*, 1992; Linder, 1992; Goyer *et al.*, 1993; Redpath and Proud, 1994). eIF-4F binds to the 5′ cap structure of mRNA and promotes unwinding of 5′ secondary structure, facilitating binding of the 43S ribosomal preinitiation complex to the mRNA. Several observations suggest that TOR could control eIF-4E. First, analyses of *CDC33* (encodes eIF-4E) and *TOR* mutants indicate that eIF-4E and TOR have remarkably similar roles. Both have essential functions required for general translation initiation (Altmann *et al.*, 1989; Kunz *et al.*, 1993; see RESULTS). Furthermore, both have an early G1-specific function and an essential function that is not G1 specific (Johnston *et al.*, 1977; Pringle and Hartwell, 1981; Brenner *et al.*, 1988; Kunz *et al.*, 1993); protein synthesis is required at several points in the cell cycle but is most limiting in G1 (Burke and Church, 1991). Second, in mammalian cells, eIF-4E is the rate-limiting protein in translation (Duncan *et al.*, 1987) and a target for regulation. Growth factors activate protein synthesis by triggering the phosphorylation and release of the eIF-4E-

Figure 8. The *UBI4-CLN3* fusion confers starvation sensitivity and an inability to arrest in G0. (A) Growth curve of NB36 cells (closed circles) expressing *UBI4-CLN3* and NB37 cells (open squares) expressing the *UBI4* 5′ region alone. (B) Viability curve of NB36 (closed circles) and NB37 (open squares) strains. Cells reached stationary phase after 3 days of growth. Strains were grown in SD medium minus leucine for the indicated times. Viability was assessed by plating 10^3 cells on YPD medium and counting colony-forming units. (C) Percentage of budded cells in cultures of NB36 (closed circles) and NB37 (open squares).

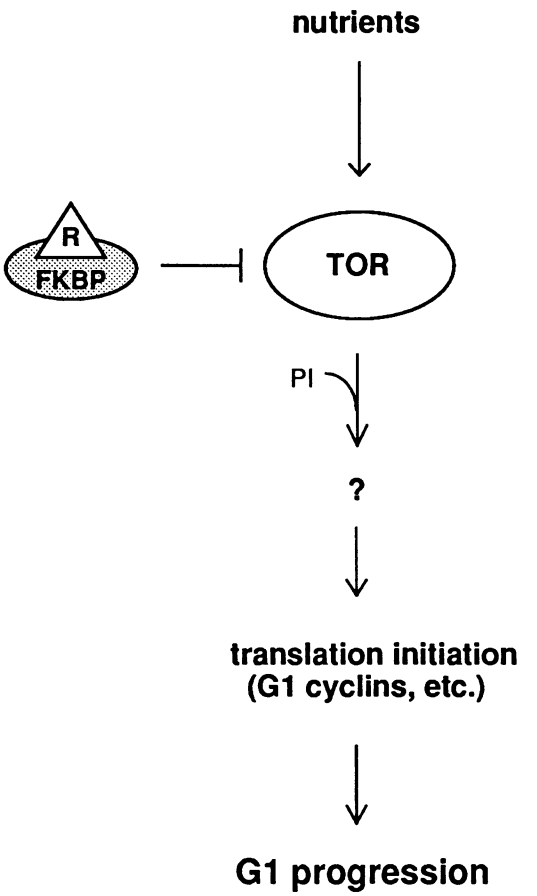


Figure 9. Model of the TOR pathway in cell cycle control. Rapamycin (R) forms a complex with FKBP to inhibit TOR (Heitman *et al.*, 1993; Kunz *et al.*, 1993). TOR is TOR1 and TOR2. PI is phosphatidylinositol. See DISCUSSION for further details. Because TOR is required for general translation (see RESULTS), the role of TOR in cell cycle control is just part of a greater role in general growth control; the model proposed here focuses exclusively on that part of TOR's role in general growth control that affects progression through the G1 phase of the cell cycle.

inhibiting factor 4E-BP1/PHAS-I (Haystead *et al.*, 1994; Hu *et al.*, 1994; Lin *et al.*, 1994; Pause *et al.*, 1994). Importantly, rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation (Beretta *et al.*, 1996). Third, in proliferating yeast and mammalian cells, eIF-4E and an associated subunit are phosphorylated and therefore potentially subject to regulation by this type of modification (Duncan *et al.*, 1987; Joshi-Barve *et al.*, 1990; Morley *et al.*, 1991; Rhoads *et al.*, 1993; Redpath and Proud, 1994; Zanchin *et al.*, 1994). Fourth, translation of UBI4 appears to have, at least, reduced dependence on eIF-4E (Brenner *et al.*, 1988). Thus, the block in translation initiation caused by loss of TOR function may be due to a down regulation of eIF-4E.

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