

The Target of Rapamycin Signaling Pathway Regulates mRNA Turnover in the Yeast *Saccharomyces cerevisiae*

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The target of rapamycin (TOR) signaling pathway is an important mechanism by which cell growth is regulated by nutrient availability in eukaryotes. We provide evidence that the TOR signaling pathway controls mRNA turnover in *Saccharomyces cerevisiae*. During nutrient limitation (diauxic shift) or after treatment with rapamycin (a specific inhibitor of TOR), multiple mRNAs were destabilized, whereas the decay of other mRNAs was unaffected. Our findings suggest that the regulation of mRNA decay by the TOR pathway may play a significant role in controlling gene expression in response to nutrient depletion. The inhibition of the TOR pathway accelerated the major mRNA decay mechanism in yeast, the deadenylation-dependent decapping pathway. Of the destabilized mRNAs, two different responses to rapamycin were observed. Some mRNAs were destabilized rapidly, while others were affected only after prolonged exposure. Our data suggest that the mRNAs that respond rapidly are destabilized because they have short poly(A) tails prematurely either as a result of rapid deadenylation or reduced polyadenylation. In contrast, the mRNAs that respond slowly are destabilized by rapid decapping. In summary, the control of mRNA turnover by the TOR pathway is complex in that it specifically regulates the decay of some mRNAs and not others and that it appears to control decay by multiple mechanisms.

INTRODUCTION

It is estimated that most of the microorganisms in the environment exist in conditions in which nutrients are limiting (Lewis and Gattie, 1991). Nutrient availability is very important in controlling cell division, growth, and physiology in microorganisms as well as in multicellular organisms. One critical response in yeast to glucose limitation is the switch from fermentation to respiration, which is termed the diauxic shift. At the diauxic shift, major changes in gene expression are induced (reviewed in Werner-Washburne *et al.*, 1996) including a general repression of translation (Fuge *et al.*, 1994) and extensive changes in the abundance of mRNAs (DeRisi *et al.*, 1997). The target of rapamycin (TOR) signaling pathway senses external nutrient availability and is involved in mediating the changes in gene expression induced at the diauxic shift (reviewed in Cutler *et al.*, 1999; Dennis *et al.*, 1999; Thomas and Hall 1999; Cardenas *et al.*, 1999). Rapamycin artificially induces a starvation-like state in yeast (Barbet *et al.*, 1996) by first forming a complex with the yeast FK506 binding protein, FKBP. This complex then binds to and represses the activity of the TOR1 and TOR2

proteins (Heitman *et al.*, 1991). The TOR signaling transduction pathway is conserved among yeast, flies, and mammals. In mammalian cells, the TOR protein homolog mTOR/FRAP/RAFT1 also coordinates nutrient and mitogenic signals to control cell growth and cell-cycle progression (reviewed in Cutler *et al.*, 1999; Thomas and Hall, 1999). The *Drosophila* TOR homolog dTOR also senses nutrient availability (Oldham *et al.*, 2000; Zhang *et al.*, 2000). The TOR proteins are protein kinases (Alarcon *et al.*, 1999) and are members of the phosphatidylinositol kinase-related kinase superfamily (Helliwell *et al.*, 1994), which includes the DNA-PK, ATM, ATR, MEC1, and TEL1 proteins, all of which regulate cell-cycle progression (reviewed in Keith and Schreiber, 1995; Kuruvilla and Schreiber, 1999). Several downstream effectors of TOR have been identified in yeast including the catalytic subunits of PP2A, TAP42p (which regulates PP2A activity), Gln3p, and Ure2p (Di Como and Arndt, 1996; Beck and Hall, 1999; Bertram *et al.*, 2000).

Levels of yeast mRNAs are dramatically altered upon entry into diauxic shift. Microarray analysis of mRNA expression in yeast shows that nearly 20% of all yeast mRNAs are down-regulated and that nearly 14% are up-regulated at least twofold upon entering the diauxic shift (DeRisi *et al.*, 1997). The treatment of yeast with rapamycin causes changes in mRNA abundance that overlap with the changes induced at the diauxic shift (Hardwick *et al.*, 1999). Some of the changes in mRNA abundance that occur when TOR signaling is inhibited are due to the regulation of transcription (Beck and Hall, 1999; Carde-

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nas *et al.*, 1999; Hardwick *et al.*, 1999; Powers and Walter, 1999). Translation also undergoes dramatic changes at diauxic shift with translation rates decreasing to 2–25% of log-phase values (Boucherie, 1985; Fuge *et al.*, 1994). Likewise, the treatment of yeast with rapamycin reduces the translation initiation rates to <50% after only 15 min and to less than 20% after 60 min of exposure (Barbet *et al.*, 1996). The translational down-regulation occurs at several levels, including decreased synthesis of rRNAs (Ju and Warner, 1994; Powers and Walter, 1999), the down-regulation of mRNAs for ribosomal proteins, translation initiation and elongation factors (DeRisi *et al.*, 1997; Powers and Walter, 1999; Shamji *et al.*, 2000), and degradation of the translation initiation factor eIF4Gp (Berset *et al.*, 1998).

The regulation of mRNA turnover continues to emerge as an important way in which cells control gene expression. Significant progress has been made in understanding how mRNA turnover is controlled in yeast. There are currently three known mRNA decay pathways in yeast, including the deadenylation-dependent decapping decay pathway, the 3'-to-5' exonucleolytic decay pathway, and the nonsense-mediated decay pathway (reviewed in Beelman and Parker, 1995; Decker, 1998; Czaplinski *et al.*, 1999). Under normal conditions, the deadenylation-dependent decapping decay pathway appears to play the most significant role in degrading mRNAs in yeast. Deadenylation proceeds first (Decker and Parker, 1993) through the action of the Ccr4 and Caf1 proteins (Tucker *et al.*, 2001). DCP1p (decapping protein 1) next removes the 7mG cap (Muhlrad *et al.*, 1994; Muhlrad *et al.*, 1995; Beelman *et al.*, 1996), which is closely followed by 5'-to-3' decay by XRN1p (exoribonuclease 1) (Hsu and Stevens, 1993; Muhlrad *et al.*, 1994; Muhlrad *et al.*, 1995).

The experiments presented here provide evidence that the TOR signaling cascade controls mRNA turnover in yeast. We demonstrate that blocking TOR signaling, either through nutrient limitation (diauxic shift) or rapamycin treatment, causes the accelerated turnover of a subset of mRNAs, while others are not effected. Furthermore, the inhibition of TOR appears to destabilize mRNAs by at least two different mechanisms.

MATERIALS AND METHODS

Yeast Strains

yRP384 (MAT α ura3–52 leu2–3112 trp1- Δ 1 his3- Δ 200 mfa1::LEU2 mfa2::URA3 rbp1–1) (Muhlrad and Parker, 1992) and yRP693 (MAT α ura3–52 leu2 rpb1–1) were used in these studies. yRP693 was constructed by converting the mating type of yRP582 (Decker and Parker, 1993) with HO endonuclease under the HO promoter. To create yCD65 (MAT α ura3–52 leu2 rpb1–1 tor1::APT), the TOR1 gene (from nucleotide-4 relative to the start codon through the stop codon) was deleted from yRP693 with the use of a PCR fragment amplified with oCD148 and oCD149 as primers and with pCH153, which contains the Tn903 APT gene flanked by the yeast PGK1 promoter and 3' end-formation sequences (Hadfield *et al.*, 1990), as a template. yCD65 was transformed with either pPW2 (TOR1–1) or pPW20 (TOR1) (Helliwell *et al.*, 1994). Yeast strains were transformed by the lithium acetate method of Gietz and Schiestl (1995), and plasmids were maintained by growth on selective media.

Plasmid Construction

The plasmids used in the MFA2M3pG transcriptional pulse-chase experiment and a corresponding plasmid bearing the WT MFA2pG gene were constructed by PCR amplification of the GAL1 promoter, MFA2 gene, and downstream flanking sequence either from

pRP410, which contains the wild-type (WT) MFA2 gene (Decker and Parker, 1993), or pRP413, which contains the MFA2 gene with three point mutations (M3) in its 3'UTR (U248C, U251G, and U282C) (Muhlrad and Parker, 1992) with oCD33 (complementary to GAL1 UAS and introduces XbaI site) and oCD34 (introduces HindIII site 720 nucleotides 3' of MFA2 start codon). Amplified fragments were inserted into the LEU2 CEN shuttle vector pRS405 (Sikorski and Hieter, 1989) that was cut with XbaI and HindIII to make pCD30 (WT) and pCD31(M3). A poly(G) tract was inserted into the 3'UTRs of both the WT and M3 MFA2 genes at position B178 (Decker and Parker, 1993). First, a BglIII site was introduced 178 nucleotides downstream of the transcription start site (B178; Muhlrad and Parker, 1992) with the use of the Quickchange Kit (Stratagene, Burlingame, CA) with oCD79 and oCD80. oCD126 and oCD127 were inserted into the B178 site to make pCD56 (MFA2pG) and pCD58 (MFA2M3pG). The plasmids used to express MFA2pG (pCD61) and MFA2M3pG (pCD62) from the endogenous MFA2 promoter were constructed by replacing the 386-bp BamHI fragment encompassing the MFA2 3'UTR in pRP264 (Muhlrad and Parker, 1992) with the corresponding fragment from pCD56 and pCD58. The plasmid used for the ARO4pG transcriptional pulse chase (pCD116) was made by first cloning the ARO4 gene from yeast chromosomal DNA by PCR with oCD152 and oCD153. The ARO4 PCR product was digested with HindIII and BamHI and ligated to HindIII/BamHI cleaved pRP22 (Heaton *et al.*, 1992) to make pCD111. The poly(G) tract was inserted into pCD111 by introducing a BglIII site into the ARO4 3'UTR with the use of the Quickchange mutagenesis kit with oCD162 and oCD163. These oligonucleotides introduce two point mutations, T 1145 G, and A 1148 C, relative to the start codon (Kunzler *et al.*, 1992). The poly(G) tract formed by hybridization of oRP126 and oRP127 was inserted at the newly created BglIII site and was checked for proper orientation by sequencing with oCD153.

Oligonucleotides

oCD33 (for transferring galactose-regulated MFA2 genes into LEU2 CEN plasmid), 5'GGGTCTAGAGTACGGATTAGAAGCCGCCG;

oCD34 (for transferring galactose-regulated MFA2 genes into LEU2 CEN plasmid), 5'GGAAGCTTCGATTCCGAAGTGGGCACCGCG;

oCD79 (for creation of BglIII site at nucleotide 178 of MFA2 mRNA), 5'CGACAACCAAGAGATCTAATCAATATCTACCC;

oCD80 (for creation BglIII site at nucleotide 178 of MFA2 mRNA), 5'GGGTAGATATTGATTAGATCTCTTGGTTGTCG;

oCD113 (detects ARO4 mRNA), 5'TTCCAGCCGACGGTTGTTGTC;

oCD114 (detects CRY1 mRNA), 5'TCTAGTACCACCGGTAGGTC;

oCD115 (detects CYS3 mRNA), 5'CGATTGCAAGATTGTGCGCG;

oCD117 (detects GRC5 mRNA), 5'GCGGCCAAACCGTGTGCGCTT;

oCD118 (detects TIF51A mRNA), 5'GAAGGAGATGGCGGCTTCTT;

oCD148 (for deletion of TOR1 gene), 5'CATTGGTAAAGTGAAACATACATCAACCGGCTAGCAGGTTTGCATCCCTCATATAAGCACGCTGGCC;

oCD149 (for deletion of TOR1 gene), 5'AAAATAAATAGTAAACAAAGCACGAAATGAAAATGACACCGCAGACTTAAATACGCTGACCC;

oCD152 (for cloning ARO4 and addition of BamHI site at 5' end), 5'GGGGGATCCCAACGATGAAATGAAAAATTTTGCTTGAA;

oCD153 (for cloning ARO4 and addition of HindIII at 3' end), 5'TAGTCGTCGATATCAAGGAATATCAACTTATGTAATGAGCTTGGG;

oCD160 (for introduction of BglIII site in ARO4 3'UTR), 5'GATGTTTTTTAATGAGATCTGTAACGTACATTCTTCTCTACC;

oCD161 (for introduction of *Bgl*III site in ARO4 3'UTR), 5'GGTAGAGGAAAGAAATGTACGTTACAGATCTCATTA A A A A C A T C;

oCD163 (for RNase H cleavage of ARO4pG), 5'GTAACT-TCTCTCTTTGTCTGAC;

oRP100 (detects 7S RNA, for correcting for differences in RNA loading on Northern blots), 5'GTCTAGCCGCGAGGAAGG;

oRP126 (for insertion of poly(G) tract) (Decker and Parker 1993), 5'GATCTAGGAATTTGGGGGGGGGGGGGGGGAATTCCT; and

oRP127 (for insertion of poly(G) tract and detection of mRNAs with poly(G) insertion), 5'GATCCAGGAATCCCCCCCCCCCCCCCCCAATCCTA.

RNA Analyses

RNA Preparation and Northern Analysis. Total RNA was isolated from frozen cell pellets, as previously described (Caponigro *et al.*, 1993). Unless otherwise noted, RNA was fractionated on 1.75% formaldehyde agarose gels run for 3.5 h at 70 V, transferred to Zeta-Probe (Bio-Rad, Richmond, CA) with 10× SSC, UV-cross-linked, and probed by standard methods. Northern analysis was quantified with the use of a Storm860 Phosphorimager (Molecular Dynamics, Eugene, OR) and ImageQuant software (Molecular Dynamics). A RNA polymerase III transcript, 7S RNA, which was detected with oRP100, was used to correct for differences in RNA loading (Caponigro *et al.*, 1993).

mRNA Half-life Analysis. mRNA decay rates were determined by thermal inactivation of *rpb1-1* with the use of a protocol modified from Herrick *et al.* (1990). Cultures of yRP384 carrying either pCD61 or pCD62 were grown in 200 ml of synthetic selective media with 2% glucose at 24°C from an OD₆₀₀ of 0.1 to an OD₆₀₀ of 0.3–0.4. Cells were harvested by centrifugation and were resuspended in 10 ml of fresh media at 24°C. The temperature of the culture was rapidly adjusted to 36°C by the addition of 10 ml of media that was preheated to 58°C, and the entire culture was transferred to a 36°C water bath. Aliquots were removed at various times, cells were harvested by centrifugation, and cell pellets were frozen on dry ice. When rapamycin was used, rapamycin (or an equivalent amount of vehicle) was added to a final concentration of 0.2 μg/ml when the culture reached an OD₆₀₀ of 0.3, the cells then were incubated for the indicated amount of time before the temperature shift was performed. Rapamycin (Sigma, St. Louis, MO) was diluted from a stock solution of 1 mg/ml in 90% ethanol and 10% Tween-20. The media used for the transcriptional shut off also contained rapamycin or vehicle. When mRNA half-life analysis was performed at the diauxic shift, 25-ml cultures were grown from an OD₆₀₀ of 1 until the cell division rate decreased relative to the rate during log-phase growth (as determined by monitoring OD₆₀₀ vs. time). Diauxic shift usually occurred at approximately OD₆₀₀ 5.0. At that point, 20 ml of culture were harvested by centrifugation. Ten milliliters of the recovered exhausted media were heated to 58°C. The cells were resuspended in the remaining 10 ml of exhausted media, and transcription was inhibited by the addition of the prewarmed exhausted media.

Analysis of the Mechanism of mRNA Decay in the Presence of Rapamycin. Transcriptional pulse-chase analysis was performed as previously described (Decker and Parker, 1993), except that the pH of the media was adjusted to 6.5 with ammonium hydroxide. Briefly, cultures of yRP693 carrying pCD58 (MFA2M3pG) or pCD116 (ARO4pG) were grown in synthetic media containing 2% raffinose to an OD₆₀₀ of 0.3–0.4, the cultures were treated with rapamycin or vehicle for 210 min (MFA2M3pG) or 60 min (ARO4pG), transcription was induced for 10 min by the addition of galactose, and transcription then was inhibited by simultaneously adding glucose and rapidly shifting the temperature of the culture to 36°C. RNase H reactions, RNA separation in 6% polyacrylamide (MFA2M3pG) or 8% (ARO4pG)/7.7 M urea gels at 300 V for 9 h

(MFA2M3pG) or 11 h (ARO4pG), and transfer to the Zeta-Probe membrane were all performed as described previously (Decker and Parker, 1993).

Analysis of the MFA2pG mRNA Decay Fragment. The amount of decay fragment relative to the amount of the full-length MFA2pG mRNA was measured in a culture of yRP384 containing pCD61 that was grown to log phase or diauxic shift, or was measured in a culture of yCD65 containing pCD56 and either pPW20 (TOR1) or pPW2 (TOR1-1) (Helliwell *et al.*, 1994) that was treated with rapamycin or vehicle for 210 min.

RESULTS

Multiple mRNAs Are Degraded Faster at Diauxic Shift Than in Log Growth

To determine whether changes in mRNA turnover were induced at diauxic shift, we compared the rates of mRNA turnover between yeast cultures in log phase or in cultures as they entered diauxic shift. We initially investigated the turnover of the WT MFA2 mRNA (MFA2pG) and a stable mutant form of this mRNA (MFA2M3pG), which carries three point mutations in its 3'UTR (see MATERIALS AND METHODS; and Muhlrud and Parker, 1992). These mRNAs were driven from the same promoter, had identical 5' leader sequences, and were marked with a poly(G) tract insert in their 3'UTRs, which does not effect stability but allow for the analysis of decay fragments (Decker and Parker, 1993). mRNA half-lives were measured with the use of a temperature-sensitive allele of RNA polymerase II, *rpb1-1*, to inhibit transcription (Nonet *et al.*, 1987). As has been shown previously (Muhlrud and Parker, 1992), the mutant MFA2M3pG mRNA was more stable than the MFA2pG mRNA at log phase, with a half-life of 12.5 min compared with 5 min for the WT control (Figure 1A). At diauxic shift, the half-life of the WT mRNA was unchanged compared with log phase. In contrast, however, at diauxic shift the mutant mRNA was destabilized approximately twofold to 5.6 min (Figure 1A). This result indicated that the decay of the mutant MFA2M3pG mRNA is accelerated at diauxic shift, whereas the WT mRNA is unaffected under these conditions.

It was important to determine whether mRNA destabilization was a general phenomenon at diauxic shift or whether this effect was unique to the MFA2M3pG mRNA. To address whether multiple mRNAs were destabilized at diauxic shift, we compared the half-lives of several mRNAs at early log phase with those at diauxic shift. We selected mRNAs to examine which are less abundant at diauxic shift compared with log phase (DeRisi *et al.*, 1997), because these mRNAs were more likely to be destabilized. These mRNAs include those for 2-dehydro-3 deoxyphosphoheptonate-aldolase (ARO4), ribosomal protein S14A (CRY1), cystathionine gamma lyase (CYS3), ribosomal protein L10 (also referred to as growth control gene 5 [GRC5]), and eukaryotic translation initiation factor 5A (TIF51A). The stable PGK1 mRNA also was analyzed because its decay at log phase has been well-studied. The ARO4, CRY1, and GRC5 mRNAs were significantly destabilized during diauxic shift (Figure 1B). In contrast

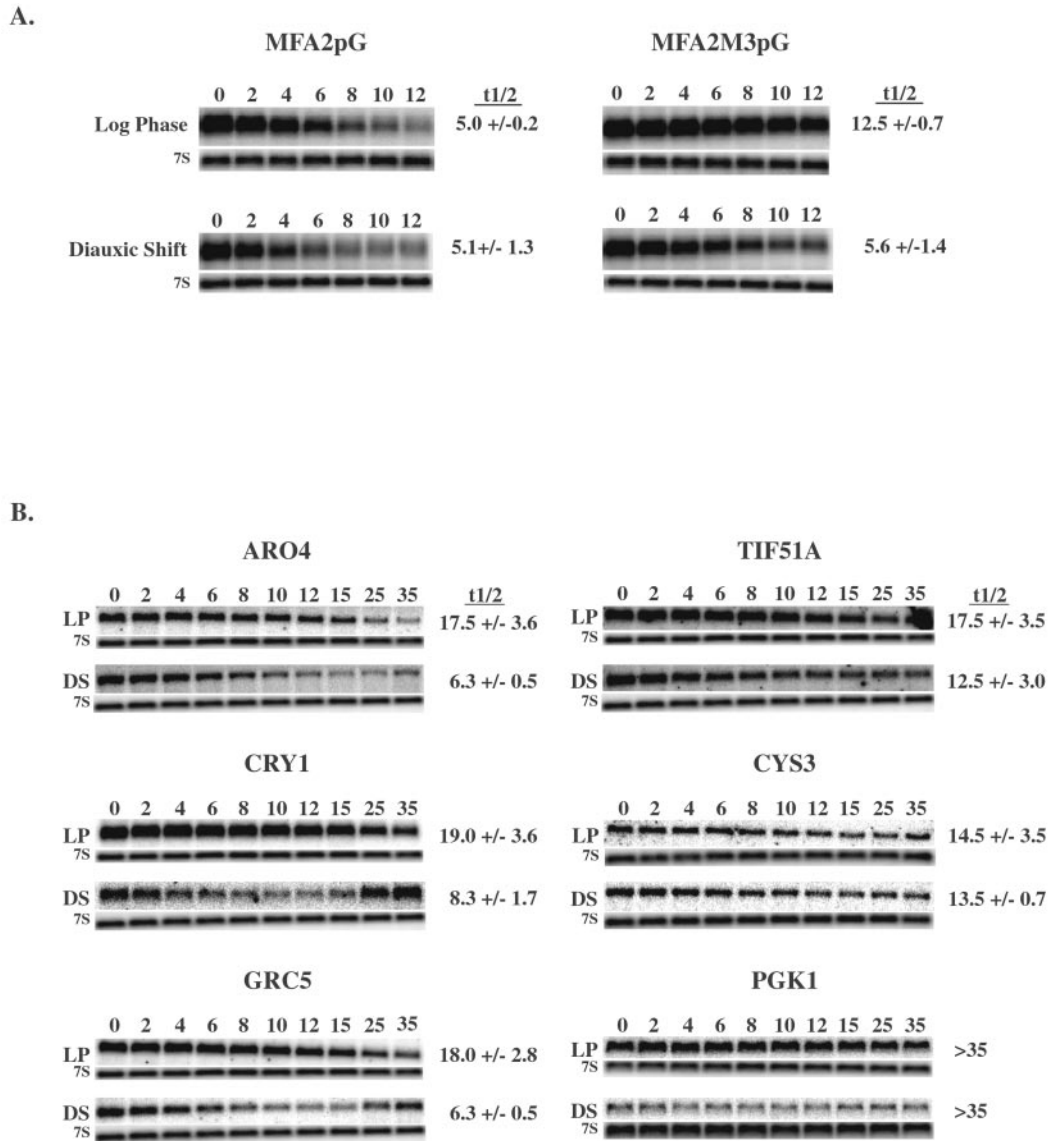


Figure 1. Multiple mRNAs are destabilized at diauxic shift. (A) mRNA half-life analysis of MFA2pG and MFA2M3pG mRNA from either log-phase or diauxic shift cultures. 5' end-labeled oRP127 was used to detect mRNAs (upper panel). The numbers above the lanes indicate the number of minutes after transcription repression. As a loading control, blots were stripped and reprobated with oRP100 to detect the RNA polymerase III 7S transcript (lower panel). (B) mRNA half-life measurements of six different mRNAs at log phase (LP) or diauxic shift (DS). The numbers above the lanes indicate the number of minutes after transcription repression. The ARO4, CRY1, GRC5, CYS3, and TIF51A mRNAs were detected with the use of 5' end-labeled oligonucleotides complementary to their coding regions (see MATERIALS AND METHODS). The PGK1 mRNA was detected with a random prime-labeled probe that is complementary to the 5' UTR and 171 nucleotides of the coding region. The oligonucleotide used to detect CRY1 mRNA is also complementary to CRY2 mRNA, which is much lower in abundance (Paulovich *et al.*, 1993). The oligonucleotide used to detect TIF51A (HYP2) is also complementary to the TIF51B (ANB1, HYP1) mRNA, which is not expressed under these aerobic conditions (Schnier *et al.*, 1991). 7S RNA again was used as a loading control (7S). In both A and B, mRNA half-life values were calculated by normalization to the 7S RNA and are the mean and SD of at least two experiments.

to the ARO4, CRY1, and GRC5 mRNAs, no significant change in half-life was detected for the TIF51A, CYS3, and PGK1 mRNAs (Figure 1B). These results, taken together with the destabilization of the MFA2M3pG mRNA, suggest that mRNA turnover is an important component of gene regulation at diauxic shift.

Blocking the TOR Signaling Pathway with Rapamycin also Induces Rapid mRNA Decay

To determine whether inhibiting TOR signaling with rapamycin caused effects on mRNA turnover that were similar to those observed at diauxic shift, log-phase yeast cultures

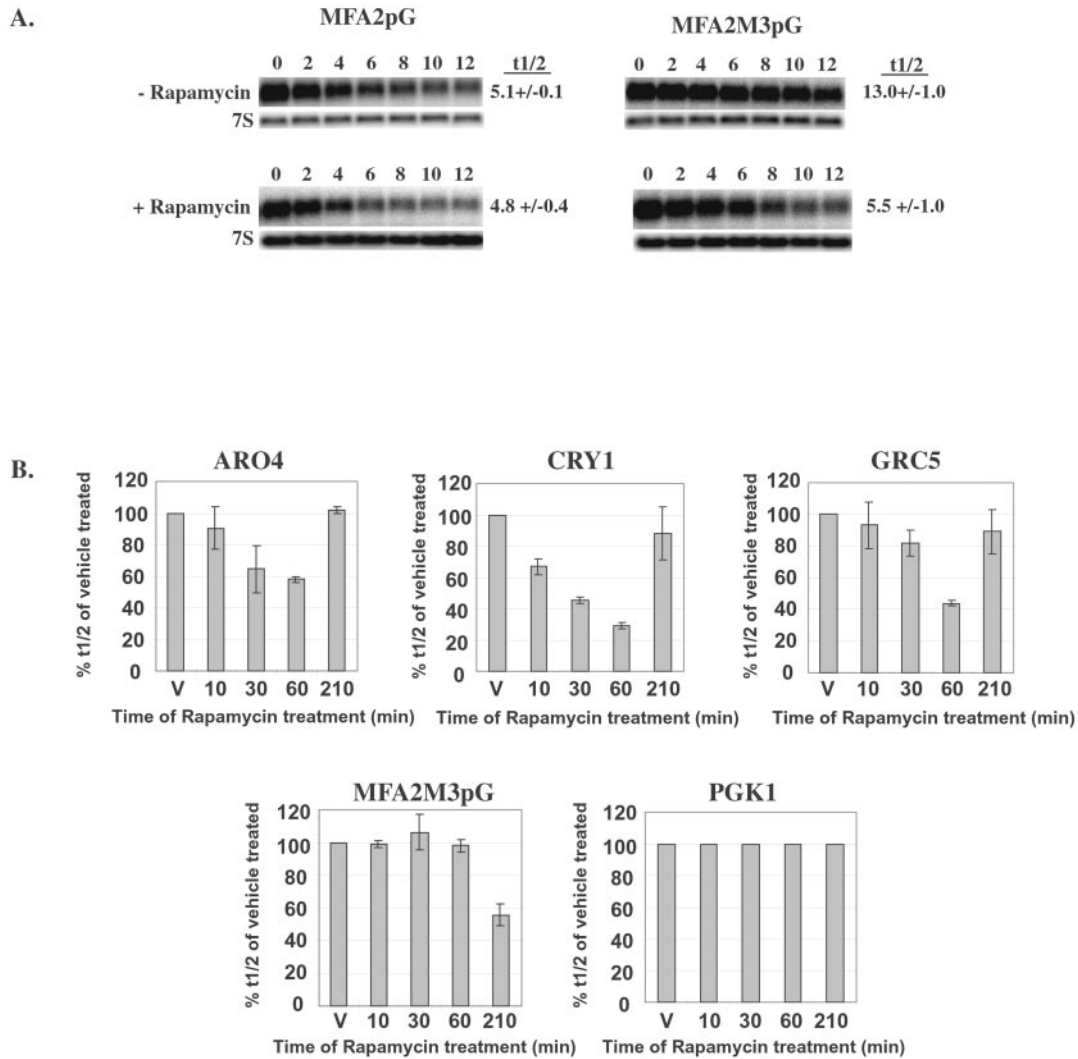
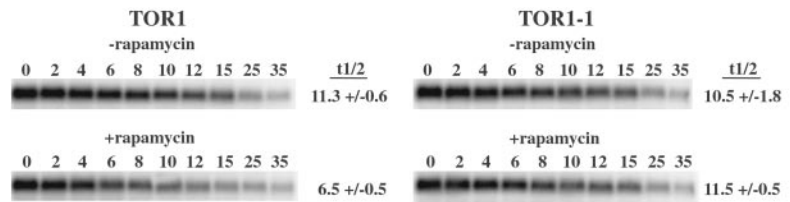


Figure 2. Rapamycin effects on mRNA turnover are similar to the turnover effects caused by diauxic shift. (A) Half-life analysis of the MFA2pG and MFA2M3pG after 210 min of exposure to vehicle (– rapamycin) or rapamycin (+ rapamycin). The number of minutes after transcription inhibition are indicated at the top of each panel. 7S RNA was used for a loading control and was detected with oRP100. Half-life values are the mean and SD of at least two independent experiments. (B) Yeast cultures were grown to early log phase then were treated with rapamycin for 10, 30, 60, or 210 min or alternatively with vehicle (V) for 210 min followed by mRNA half-life analysis. RNA analysis was performed with the same probes that were used in Figure 1B and were normalized by comparison to the 7S RNA. mRNA half-lives at each rapamycin time point are presented as a percentage of the half-life in the vehicle-treated control for that mRNA. The control half-lives were as follows: ARO4, 17.3 ± 1.8 min; CRY1, 26.5 ± 0.7; GRC5, 16.7 ± 2.1; MFA2M3pG, 16 ± 2.5 min; and PGK1, >25 min. The results for all time points are the mean and SD of at least two independent experiments.

expressing either the MFA2pG or MFA2M3pG mRNAs were treated with either rapamycin in vehicle or with vehicle alone for 210 min. Treatment with vehicle had no effect on the turnover of the MFA2pG or MFA2M3pG mRNAs (compare Figure 1A log phase and Figure 2A rapamycin). The treatment of log-phase cultures with rapamycin induced similar effects on mRNA turnover compared with those at diauxic shift. The MFA2M3pG mRNA again was destabilized approximately twofold to 5.5 min, while the turnover of the MFA2pG mRNA remained unchanged (Figure 2A). This result suggested that turnover of the MFA2M3pG mRNA is regulated by the TOR signaling pathway.

We next investigated the effects of rapamycin on mRNA turnover over a time course of rapamycin treatment. The half-lives of the ARO4, CRY1, GRC5, MFA2M3pG, and PGK1 mRNAs were measured after 10, 30, 60, and 210 min of rapamycin treatment and were compared with the half-life that was observed after treatment with vehicle for 210 min. Three distinctly different mRNA turnover phenotypes were observed after rapamycin treatment (Figure 2B). The ARO4, CRY1, and GRC5 mRNAs were rapidly destabilized after only 10–30 min of exposure to rapamycin and were further destabilized by 60 min of treatment. Surprisingly, however, at 210 min of rapamycin treatment, the ARO4,

Figure 3. Rapamycin does not destabilize mRNAs in the presence of a rapamycin-resistant allele of the TOR1 kinase. The stability of the MFA2M3pG mRNA in a *tor1Δ* strain carrying either a plasmid bearing the WT TOR1 gene (*TOR1*) or the rapamycin-resistant TOR1-1 allele (*TOR1-1*) after treatment with rapamycin (+ rapamycin) or with vehicle alone (- rapamycin) for 210 min. The numbers above each lane indicate the number of minutes after transcription was repressed. The half-life values are the mean and SD of at least three experiments for each strain.



CRY1, and GRC5 mRNAs were restabilized close to their respective control half-lives. For example, the half-life of the ARO4 mRNA was 17.3 min in control cells. By 10 min of rapamycin treatment, the half-life was reduced to 15.6 min (90% of control). The ARO4 mRNA was destabilized even more after 30 min of treatment to a half-life of 10.9 min (58% of control) and remained destabilized after 60 min of rapamycin treatment. At 210 min of exposure to rapamycin, the ARO4 mRNA half-life restabilized to 17.5 min. In sharp contrast, the MFA2M3pG mRNA was not destabilized by 10, 30, or 60 min of rapamycin treatment but was only destabilized by 210 min of exposure to the drug. Finally, similar to what was observed at diauxic shift, no half-life change was detected for the PGK1 mRNA after any length of rapamycin treatment. Although the stability of mRNAs responded with different kinetics to rapamycin, the most important observation from these results is that, similar to the situation at diauxic shift, rapamycin also destabilizes mRNAs. Furthermore, rapamycin destabilized the same mRNAs that were destabilized at diauxic shift (ARO4, CRY1, GRC5, and MFA2M3pG) and did not affect the stability of the mRNAs that had not been altered at diauxic shift (PGK1, MFA2pG).

Rapamycin Does Not Induce Accelerated mRNA Turnover in Rapamycin-resistant Yeast

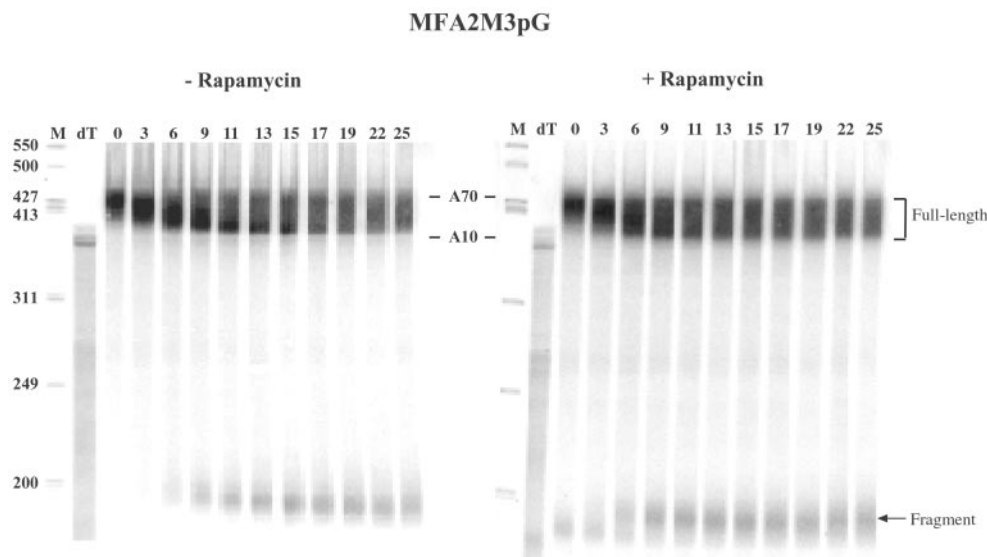
To verify that rapamycin treatment accelerated mRNA turnover via the TOR signaling pathway, rapamycin-resistant mutants were exposed to the drug and were tested for accelerated turnover. Rapamycin resistance can be conferred to yeast by the introduction of the TOR1-1 allele (Helliwell *et al.*, 1994). The TOR1-1 allele is dominant at 24°C but is recessive at 36°C, which is the temperature needed to inactivate the *rpb1-1* allele. Therefore, to ensure that the TOR1-1 allele would be effective during half-life measurements, it was necessary to delete the chromosomal TOR1 allele and introduce either the TOR1 or TOR1-1 allele on a plasmid (Helliwell *et al.*, 1994). The half-life of the MFA2M3pG mRNA was measured in TOR1 and TOR1-1 cell lines after 210 min of vehicle or rapamycin treatment (Figure 3). The MFA2M3pG mRNA was stable in both TOR1 and TOR1-1 cells that were treated with vehicle. The mRNA was destabilized by rapamycin treatment in TOR1 cells, however, rapamycin treatment of the TOR1-1 cells did not result in the destabilization of the MFA2M3pG mRNA. Furthermore, a strain carrying a deletion of the *FPR1* gene, which encodes the FK506 binding protein (FKBP) required for rapamycin to inhibit the TOR proteins (Heitman *et al.*, 1991), was also resistant to the destabilizing effect of rapamycin (data not shown). From these results, we conclude that the mRNA destabilizing effects mediated by rapamycin are due to the inhibition of the TOR signaling pathway.

Rapamycin Destabilizes MFA2M3pG mRNA by Acceleration of Decapping

To begin to understand how the TOR signaling pathway regulates mRNA decay, it was important to determine whether rapamycin treatment accelerated the deadenylation-dependent decapping mechanism or induced an alternative decay mechanism. Transcriptional pulse-chase experiments allow the analysis of deadenylation, decapping, and the directionality of mRNA decay (Decker and Parker, 1993; Muhlrud *et al.*, 1994) and, thus, were used to determine the mechanism by which mRNAs are degraded after rapamycin treatment.

To determine how prolonged exposure to rapamycin (210 min) destabilized the MFA2M3pG mRNA, a pulse of newly synthesized mRNA was created, then transcription was rapidly repressed (see MATERIALS AND METHODS). Deadenylation and decapping of these newly synthesized transcripts were monitored by removing aliquots at various time intervals and analyzing the MFA2M3pG mRNA (Figure 4). The length of the poly(A) tail on the mRNA was determined by comparing the size of the mRNA at any given time to the size of the mRNA that has had its poly(A) tail removed by treatment with oligo dT and RNase H (lane dT, Figure 4). It is important to follow through the time course the pulse of mRNA present at 0 min, which, at later time points, can be detected above the uniform background that results from residual transcription.

In both the control and rapamycin-treated cells, the MFA2M3pG mRNA decayed via the deadenylation-dependent mechanism. This is evident because there is not a loss of intensity in the pulse of mRNA until the transcripts have been deadenylated. In addition, a decay fragment accumulates after deadenylation that is the correct size to be produced by decapping and 5'-to-3' digestion up to the poly(G) structure at the 3' end of the mRNA (Decker and Parker, 1993; Muhlrud *et al.*, 1994). Furthermore, in a *dcp1Δ* cell line, the turnover of the MFA2M3pG mRNA was not accelerated by rapamycin treatment (data not shown), indicating that accelerated turnover in response to rapamycin is still dependent on decapping and, therefore, proceeds via the deadenylation-dependent decay mechanism. In both vehicle-treated and rapamycin-treated cells, the pulse of mRNA deadenylates from 0-9 min, thus, there is no significant increase in the rate of deadenylation after rapamycin treatment. The most striking difference in the decay of the MFA2M3pG mRNA after rapamycin treatment, as compared with control cells, is the rapid disappearance of the pulse of mRNA after deadenylation. The pulse of MFA2M3pG mRNA in the control cells is not completely degraded until between 15 and 17 min, whereas in the rapamycin-treated cells the pulse of mRNA disappears by 9



is marked by A70 and A10 indicators. The marker (M) lane contains 5' end-labeled *HinfI* $\phi\chi$ 174 DNA, and sizes are indicated to the left of the figure.

or 11 min. The rapid degradation of the deadenylated mRNA in the presence of rapamycin is most likely due to accelerated decapping since once an mRNA is decapped it is immediately degraded by the XRN1 exonuclease. We have found no evidence that the alternative 3'-to-5' decay pathway or other decay mechanisms are induced by rapamycin or at diauxic shift (data not shown). Therefore, the inhibition of TOR signaling primarily destabilizes the MFA2M3pG mRNA by inducing rapid decapping.

ARO4 mRNA Is Destabilized by a Prematurely Short Poly(A) Tail

The ARO4 mRNA was used as a model to determine how rapamycin effected the turnover of mRNAs after shorter intervals (60 min) of rapamycin treatment. To perform transcriptional pulse-chase analysis on the ARO4 mRNA, the ARO4 gene was first cloned under the control of a galactose-regulated promoter and a poly(G) tract was inserted into its 3'UTR (ARO4pG) (see MATERIALS AND METHODS). Like the MFA2M3pG mRNA, the ARO4pG mRNA from control cells degraded through the deadenylation-dependent decapping pathway (Figure 5). In our analysis, we focused on the major ARO4pG mRNA species, however, a slightly longer transcript that results from the use of an alternate 3' end signal is also present (Kunzler *et al.*, 1992). The most obvious difference between mRNAs from control cells and rapamycin-treated cells was that the ARO4pG mRNA had a prematurely short poly(A) tail after rapamycin treatment. In vehicle-treated cells, the pulse of mRNA had poly(A) tails 50–10 adenylate residues long, whereas in the rapamycin-treated cells, the mRNA at 0 min had tails of only 10–0 adenylate residues (in Figure 5 compare the dT lane to the 0 min lane). Due to the extremely short poly(A) tails that were present after rapamycin treatment, it was not possible to measure deadenylation in the rapamycin-treated cells, however, the observed decay fragment is identical to that in the control

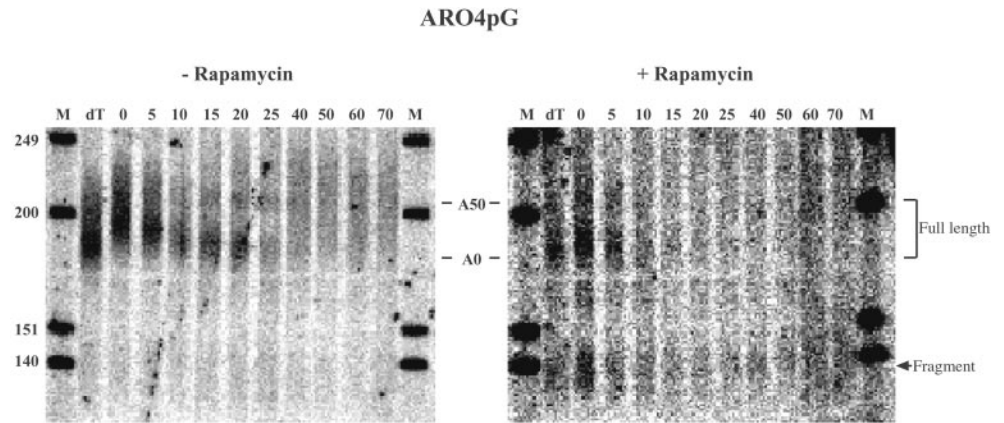
Figure 4. Rapamycin destabilizes the MFA2M3pG mRNA by the acceleration of decapping. Yeast cells expressing MFA2M3pG were treated with vehicle alone (– rapamycin) or rapamycin (+ rapamycin) for 210 min followed by transcriptional pulse-chase analysis of the MFA2M3pG mRNA. The dT lane contains RNA from the 0 min time point, hybridized to oligo(dT) and treated with RNaseH to remove the poly(A) tail. Time points (in minutes) taken after the repression of transcription are indicated at the top. The full-length and fragment species were detected with the use of oRP127 as a probe. The locations of the fragment and full-length species are indicated to the right of the figure, and the poly(A) tail length

cells and is the size expected from 5'-to-3' decay. Furthermore, in a *dcp1* Δ strain, rapamycin was not able to stimulate rapid decay of the ARO4 mRNA (data not shown), which indicates that decapping was still a prerequisite for decay. The decapping rate can be compared between the vehicle-treated cells and the rapamycin-treated cells by examining the time required to degrade the deadenylated mRNA. In the control cells, the ARO4pG mRNA reaches an oligo(A) tail by 15 min and the RNA was completely degraded ~10 min after deadenylation was complete. In rapamycin-treated cells, the ARO4pG mRNA already had an oligo(A) tail at 0 min and was degraded by 10 min. The similarity between the time needed to degrade the oligo(A) species in the control and rapamycin-treated cells suggests that decapping is not a significant factor in the destabilization of the ARO4 mRNA by rapamycin. Therefore, the ARO4pG mRNA is destabilized after rapamycin treatment by the mRNA having a prematurely short poly(A) tail.

Diauxic Shift and Rapamycin Treatment Increase the Accumulation of 5'-to-3' Decay Intermediates

In addition to changes in mRNA turnover, we observed another effect on mRNA metabolism when TOR signaling was inhibited. As shown in Figure 6, the amount of the decay fragment generated by 5'-to-3' exonucleolytic decay of the MFA2pG mRNA increased relative to the amount of the full-length mRNA at diauxic shift and after rapamycin treatment in TOR1 cells. This results in an increase in the ratio of the fragment to the full-length mRNA (F/FL). The amount of the MFA2M3pG and ARO4pG mRNA decay fragments also increased at diauxic shift and/or after rapamycin treatment (data not shown). The accumulation of fragment is due, at least in part, to inhibition of the TOR signaling pathway given that the MFA2pG decay fragment did not accumulate to the same extent in TOR1-1 cells treated with rapamycin as compared to TOR1 cells (Figure 6). The

Figure 5. The ARO4pG mRNA is destabilized after rapamycin treatment by having a prematurely short poly(A) tail. Transcriptional pulse-chase analysis of the ARO4pG mRNA after treatment with either vehicle (– rapamycin) or rapamycin (+ rapamycin) for 60 min is shown. In each sample, the ARO4pG mRNA was cleaved with oCD163 and RNase H to resolve the length of its poly(A) tail. The poly(A) tail was removed by treatment with oCD163, oligo dT, and RNase H (dT lane). Full-length and fragment species were detected with oRP127 and are indicated at the right of the figure. Poly(A) tail length is marked by A50 and A0 indicators. The marker (M) lane contains 5' end-labeled *Hinfl* ϕ X174 DNA, and sizes are indicated to the left of the figure.



accumulation of decay fragments is not solely due to faster degradation of the corresponding full-length mRNA given that the half-life of the MFA2pG mRNA is not accelerated when TOR signaling is inhibited. Another possible explanation for the increased amount of fragment is that turnover of the fragment is reduced when TOR is inhibited. The decay fragments produced by the deadenylation-dependent decapping pathway are degraded by the 3' to 5' exosome decay pathway (Anderson and Parker, 1998). However, the accumulation of the MFA2pG fragment is not due to a decrease in the rate of 3' to 5' degradation because its half-life is the same in the presence or absence of rapamycin (data not shown). One alternative explanation is that during log phase growth, 5' to 3' exonucleolytic digestion stalls at the poly(G) block on only a fraction of the MFA2pG transcripts and that inhibition of the TOR signaling pathway leads to a more efficient blockage. Perhaps, for example, the processivity of the XRN1p exonuclease may be reduced after rapamycin treatment or an RNA helicase activity that aids XRN1p digestion through the poly(G) structure could be downregulated.

DISCUSSION

The TOR Signaling Pathway Regulates mRNA Turnover in Response to Nutrient Availability

We provide three pieces of evidence that indicate that the TOR signaling pathway controls mRNA turnover in yeast. First, several mRNAs were destabilized at diauxic shift, a state of limited nutrients that is sensed by the TOR signaling pathway. Second, rapamycin induced almost identical effects on mRNA turnover as those observed at diauxic shift. Finally, the observation that the rapamycin-resistant TOR1–1 allele or the deletion of FKBP reversed the rapamycin-induced changes in mRNA turnover directly indicates that mRNA turnover is regulated by the TOR signaling pathway.

Significant, widespread changes in mRNA abundance occur at diauxic shift and after TOR signaling has been blocked by rapamycin (DeRisi *et al.*, 1997; Hardwick *et al.*, 1999; Shamji *et al.*, 2000). The fact that the majority of the mRNAs that we examined that are down-regulated at diauxic shift

were destabilized strongly suggests that the regulation of mRNA turnover by TOR plays an important role in controlling yeast gene expression in response to nutrient limitation. Although the TOR signaling pathway has been demonstrated to regulate the transcription of specific genes, in particular genes for ribosomal proteins and proteins involved in nitrogen catabolite repression (Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Powers and Walter, 1999), our observation that the CRY1 and GRC5 ribosomal protein mRNAs are destabilized illustrates that the regulation of both transcription and stability contribute to the overall changes in mRNA abundance seen when TOR signaling is blocked. Given the conservation of the TOR signaling pathway, the regulation of mRNA turnover may be an important mechanism in controlling the balance between cell growth and proliferation in multicellular eukaryotes as well. Indeed, rapamycin treatment of mammalian cells has been shown to destabilize the interleukin 3 and cyclin D1 mRNAs (Banholzer *et al.*, 1997; Hashemolhosseini *et al.*, 1998).

Previous research (Jona *et al.*, 2000) concluded that mRNAs were stabilized by the rapid withdrawal of glucose from the growth media. This finding would seem to be in direct contrast to the results presented in this report. However, recent data illustrate that rapid depletion of glucose results in cellular responses that are not TOR-dependent (Ashe *et al.*, 2000). Therefore, the stabilization of mRNAs after rapid glucose depletion is likely to be due to a mechanism that is different from what we have observed. It should also be noted that, unlike the destabilization of mRNAs that we have observed at diauxic shift when glucose becomes limiting and cells switch to respiration, the SDH1 and SDH2 mRNAs are stabilized when cells are grown in carbon sources that require respiration and are destabilized in the presence of glucose (Lombardo *et al.*, 1992; Cereghino *et al.*, 1995). Whether TOR is involved in the regulation of the stability of these mRNAs remains to be determined.

The TOR Signaling Pathway Regulates the Deadenylation-dependent Decapping Pathway

Two general possibilities for how the blocking of TOR could destabilize mRNAs are that it could accelerate the decay

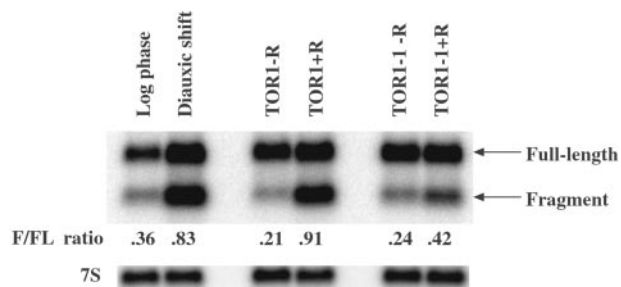


Figure 6. Diauxic shift and rapamycin treatment cause the accumulation of a 5'-to-3' decay intermediate without changing the rate of 3'-to-5' decay. Analysis of the full-length MFA2M3pG mRNA and its decay fragment at log phase, diauxic shift, or in a *tor1Δ* strain carrying either the WT *TOR1* gene or the rapamycin-resistant TOR1-1 allele after treatment with rapamycin (+ R) or vehicle alone (- R) for 210 min is shown. The MFA2M3pG mRNA and the decay fragment were detected with oRP127. Full-length and fragment species are indicated on the right. The ratio of the fragment to the full-length mRNA (F/FL) is indicated below each lane. The membrane was stripped and rehybridized with oRP100 to detect the 7S RNA as a loading control (bottom panel, 7S).

pathways that are active during log phase or it could induce a new decay mechanism. From our transcriptional pulse-chase analyses, we conclude that the TOR signaling pathway regulates mRNA turnover by controlling the predominant log-phase decay mechanism, the deadenylation-dependent decapping pathway. Furthermore, TOR inactivation appears to accelerate the turnover of mRNAs by the deadenylation-dependent decapping pathway through at least two different mechanisms. This idea was first suggested by the difference in the rate of response of particular mRNAs to rapamycin. Moreover, the fact that the MFA2M3pG mRNA became destabilized at a time when the other mRNAs were being restabilized is most consistent with the turnover of the MFA2M3pG mRNA being affected by a different mechanism than the other mRNAs. Finally, the transcriptional pulse-chase analysis indicated that the MFA2M3pG mRNA was destabilized primarily by the acceleration of decapping. In contrast, the ARO4pG mRNA was destabilized primarily by a prematurely short poly(A) tail, which ultimately contributes to the accelerated 5'-to-3' decay by shortening the time necessary to reach the oligo(A) tail, which is required for decapping to occur. Therefore, to understand how TOR activity regulates mRNA decay it will be necessary to elucidate how both decapping and poly(A) tail metabolism are controlled.

How Are Decapping and Poly(A) Tail Length Controlled by the TOR Signaling Pathway?

mRNA decapping could be controlled by TOR at several levels. One potential target of the TOR pathway is the decapping protein itself, DCP1p, since it is a phosphoprotein (LaGrandeur and Parker, 1998), although it is not known whether its phosphorylation state affects its activity. In addition to DCP1, many other genes such as *MRT4*, *GRC5*, *TIF51A*, *PAB1*, and *EDC2* are known to effect the deadenylation-dependent decapping pathway (Caponigro and Parker, 1995; Zuk and Jacobson, 1998; Zuk *et al.*, 1999;

Dunckley *et al.*, 2001). The mRNA levels for these genes are regulated at diauxic shift and/or by rapamycin treatment (DeRisi *et al.*, 1997; Hardwick *et al.*, 1999; Shamji *et al.*, 2000). However, of these five genes, the up-regulation of the mRNA encoding EDC2p, which enhances decapping, is most consistent with the increase in decapping that we have observed. Another mechanism by which the TOR signaling pathway could control mRNA decapping is indirectly through regulating mRNA translation. This idea is based on the observation that, in general, decreased translation initiation results in the destabilization of mRNAs (Muhlrad *et al.*, 1995; LaGrandeur and Parker, 1999; Schwartz and Parker, 1999). It is possible that decapping is accelerated indirectly by TOR inhibition since translation is dramatically reduced at diauxic shift and after rapamycin treatment (Boucherie, 1985; Fuge *et al.*, 1994; Barbet *et al.*, 1996). In particular, several of the translation initiation factors that are believed to protect the 7mG cap from DCP1p (Schwartz and Parker, 2000) are down-regulated at diauxic shift or after rapamycin treatment (DeRisi *et al.*, 1997; Berset *et al.*, 1998; Powers and Walter, 1999; Shamji *et al.*, 2000). If changes in translation are responsible for the observed increase in decapping, then it will be important to understand how mRNAs such as the PGK1 transcript, which have been shown to be destabilized when translation initiation is reduced (Muhlrad *et al.*, 1995; Schwartz and Parker, 1999; LaGrandeur and Parker, 1999), are not affected when TOR is inhibited.

The presence of prematurely short poly(A) tails on mRNAs when TOR is inhibited could result from the extreme acceleration of cytoplasmic deadenylation or alternatively from the loss of normal polyadenylation. In either case, TOR signaling is likely to regulate poly(A) tail length directly, given that the ARO4 mRNA begins to be destabilized after only 10 min of rapamycin treatment. If TOR regulates deadenylation, then the major cytoplasmic deadenylation factors, Caf1p and Ccr4p (Tucker *et al.*, 2001), and the PUF proteins that have been found to control deadenylation in a mRNA-specific manner (Olivas and Parker, 2000) are potential targets of regulation. If TOR controls the length of the nascent poly(A) tail, any of the multitude of factors involved in polyadenylation could be regulated, however, two particularly good candidates are the PAN2/PAN3 nuclease, which is believed to trim new poly(A) tails in the nucleus to a mRNA-specific length (Brown and Sachs, 1998), and PBP1p, which is required for the addition of long poly(A) tails *in vitro* and is down-regulated at late log phase (Mangus *et al.*, 1998).

Two important questions arise from our results. Why are some mRNAs destabilized when TOR signaling is inhibited while others are unaffected? And, what determines whether a particular mRNA is susceptible to enhanced decapping or to alterations to poly(A) tail metabolism? It is especially intriguing that TOR inhibition somehow suppresses the stabilizing effects of the mutations located in the instability elements of the MFA2 mRNA. Thus, investigation into how TOR regulates mRNA turnover is not only essential to understanding how gene expression is controlled on a global level in response to nutrient availability, but it may also lead to a better understanding of how specific elements within mRNAs control mRNA stability.

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