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Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family

(protein synthesis/translational control/ribosomal protein S6 phosphorylation/p70^{s6k}/p85^{s6k})

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ABSTRACT **The immunosuppressant rapamycin blocks p70^{s6k}/p85^{s6k} activation and phosphorylation of 40S ribosomal protein S6 in Swiss 3T3 cells. The same net result is obtained when the macrolide is added 3 hr after serum stimulation. In stimulated cells p70^{s6k}/p85^{s6k} inactivation is achieved within minutes, whereas S6 dephosphorylation requires 1–2 hr, supporting the concept that S6 dephosphorylation results from kinase inactivation. In parallel, rapamycin treatment causes a small, but significant, reduction in the initiation rate of protein synthesis, as measured both by [^{35}S]methionine incorporation into protein and by recruitment of 80S ribosomes into polysomes. More striking, analysis of individual mRNA transcripts revealed that rapamycin selectively suppresses the translation of a family of mRNAs that is characterized by a polypyrimidine tract immediately after their N^7-methylguanosine cap, a motif that can act as a translational modulator. This family includes transcripts for ribosomal proteins, elongation factors of protein synthesis, and proteins of as-yet-unknown function. The results imply that (*i*) 40S ribosomes containing phosphorylated S6 may selectively recognize this motif or proteins which bind to it and (*ii*) rapamycin may inhibit cell growth by blocking S6 phosphorylation and, thus, translation of these mRNAs.**

The immunosuppressive macrolide rapamycin either reduces or abolishes the rate at which cells enter S phase and subsequent proliferation, depending on the cell type (1). This effect is elicited through the association of rapamycin with an intracellular FK506- and rapamycin-binding protein, termed FKBP (1, 2). Recently, rapamycin was shown to block the mitogen-induced activation of p70^{s6k}/p85^{s6k} and to rapidly inactivate the kinase in mitogen-stimulated cells (3–6). This inhibition is apparently not exerted on the kinase but is exerted on a component involved in controlling its activity (3–6). The effect of rapamycin appears selective, as the macrolide does not block the activation of other kinases—such as p74^{raf}, p42^{mapk}, or p90^{rsk}—that are also triggered to act within minutes of mitogen addition (3–6). Activation of p70^{s6k}/p85^{s6k} itself has been associated with the phosphorylation of four residues that exhibit Ser/Thr-Pro motifs and are located within 14 residues of one another in a putative autoinhibitory domain (7). However, rapamycin does not abolish p70^{s6k}/p85^{s6k} activity through altering the phosphorylated state of these sites but works through a specific set of sites, which apparently turn over slowly or not at all (8).

The p70^{s6k}/p85^{s6k} represent two isoforms of the same enzyme, derived by differential splicing from a common gene (9) and whose target is 40S ribosomal protein S6 (10). The p85^{s6k} sequence is identical to that of p70^{s6k}, except for a 23-aa extension at its amino terminus, which contains a nuclear-targeting sequence (9), consistent with the finding of S6 phosphorylation in the nucleus after mitogenic stimulation (11). The five phosphorylation sites within S6 reside in a 15-aa fragment at the carboxyl terminus and are phosphorylated in a specific order (10). The kinase is highly selective for S6, exhibiting a K_m of 0.25 μM (12), and *in vitro* phosphorylates four and, possibly, the fifth site observed *in vivo* (13). By using several approaches, S6 has been mapped to the tRNA-mRNA-binding site of the 40S ribosome (14) and in the phosphorylated state has been implicated in the activation of protein synthesis, as well as in alterations in the pattern of translation (10). Because rapamycin blocks S6 phosphorylation, presumably by inhibiting p70^{s6k}/p85^{s6k} (3), its inhibitory effects on cell growth may be through inhibiting S6 phosphorylation and thus, protein synthesis.

We examined the effect of rapamycin on p70^{s6k}/p85^{s6k} activity and S6 phosphorylation in quiescent and serum-stimulated cells. Then we analyzed the role of the macrolide in the activation of specific mRNA transcripts. Our results show that rapamycin has an inhibitory effect on the activation of protein synthesis and, more importantly, that this effect is through suppressing translation of a family of mRNAs known to be under translational control.

EXPERIMENTAL PROCEDURES

Cell Culture and [^{35}S]Methionine Labeling. Swiss mouse 3T3 cells were seeded, maintained, and quiesced on 15-cm tissue culture plates as described (15). Rapamycin (20 mg/ml in ethanol) was diluted 1:100 in medium before being added to cell cultures at the indicated times to a final concentration of 20 ng/ml. For [^{35}S]methionine-labeling studies, cells were seeded at 6×10^4 cells per 35-mm tissue culture plate, as described (16, 17), except that the volume was reduced to 0.75 ml during labeling for the indicated times, and 20 μCi of [^{35}S]methionine (Amersham) (1 Ci = 37 GBq) was added per plate. The amount of [^{35}S]methionine incorporated into protein was essentially determined as described (18).

Analysis of mRNA. Preparation of cell extracts, gradient centrifugation, fractionation of polysome profiles, and analysis of mRNA by either Northern blot or S1 protection-hybridization were done as described (19).

Radioactive Labeling of Probes. The elongation factor 1α (eEF-1α) oligonucleotide probe is complementary to the first 57 coding bases of mouse eEF-1α mRNA (GenEMBL accession no. X13661), the β-actin oligonucleotides probe is complementary to the first 57 bases of the 3′ untranslated region of mouse β-actin mRNA (GenEMBL accession no. X03765), and the eEF-2 oligonucleotide probe is complementary to bases 60–127 of the partial mouse sequence (GenEMBL accession no. M76131). The oligonucleotide probes were 5′ end-labeled by using T4 kinase (Boehringer Mannheim), as described (19). The S6 probe is a complete mouse S6 cDNA (20) labeled by using a random oligomer-priming kit (Boeh-

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Abbreviation: eEF-1α, elongation factor 1α.

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ringer Mannheim). All labeled probes were purified on G-25 Sephadex spun columns (21).

RESULTS

$p70^{s6k}/p85^{s6k}$ Activation and S6 Phosphorylation. The effect of rapamycin on the serum-induced activation of $p70^{s6k}$ and $p85^{s6k}$ after immunoprecipitation from cell extracts of $p70^{s6k}/p85^{s6k}$ with the M1 antibody (22) or only $p85^{s6k}$ with the C3-specific antibody (9) was measured in a kinase assay using 40S ribosomal subunits as substrate (9, 22). In both cases $p70^{s6k}/p85^{s6k}$ or $p85^{s6k}$ activation was abolished (Fig. 1 *A* and *B*, lanes 1–3, respectively). In the presence of the vehicle ethanol no inhibitory effect on kinase activity was observed (Fig. 1 *A* and *B*, lanes 4 and 5). If, instead, rapamycin is added 3 hr after serum stimulation, rapid inactivation of $p70^{s6k}/p85^{s6k}$ occurs within 10 min (data not shown), an effect maintained for at least 2 hr after rapamycin treatment (Fig. 1 *A* and *B*, lanes 6–8) and consistent with earlier findings (3–6). In previous studies the block of S6 phosphorylation was assumed to be exerted through inhibition of $p70^{s6k}/p85^{s6k}$ activity, although no data were provided regarding the rapamycin effect on S6 phosphorylation in stimulated cells (3, 6). In quiescent cells two-dimensional gel electrophoresis disclosed that S6 was largely in the dephosphorylated state; minor amounts of phosphate were in derivates a and b, containing 1 or 2 mol of phosphate, respectively (Fig. 1*C1*). After serum stimulation for 3 hr, most S6 was found in the highly phosphorylated derivates d and e, containing 4 or 5 mol of phosphate, respectively (Fig. *C3*). Pretreatment with rapamycin blocked this mobility shift and caused further dephosphorylation of the protein, with almost complete loss of derivates a and b (Fig. *C2*). If, instead, rapamycin is added to cells 3 hr after serum stimulation, a slow net dephosphorylation of ribosomal protein S6 occurs, with the protein only returning to its native dephosphorylated position after 120 min (Fig. *C 4–6*). The results support the hypothesis that S6 dephosphorylation is a consequence of $p70^{s6k}/p85^{s6k}$ inactivation, rather than from induction of a rapamycin-specific S6 phosphatase.

Protein Synthesis. Stimulation of quiescent cells to proliferate leads to a 3- to 4-fold increase in the protein-synthesis rate (10). To test whether rapamycin inhibits this increase in protein synthesis, the amount of [^{35}S]methionine incorporated into nascent protein was determined in cells stimulated with serum and either with rapamycin or the vehicle alone (Fig. 2*A*). Results show that rapamycin causes a small, but significant, inhibition of serum-stimulated protein synthesis. The serum-stimulated increase in protein synthesis (Fig. 2*A*) is exerted at the level of initiation (10), which is easily monitored as the recruitment of inactive 80S ribosomes into actively translating polysomes (19). To examine whether rapamycin was acting at the level of initiation rather than, for instance, at elongation, the recruitment of 80S ribosomes was monitored in the presence and absence of the macrolide. Analytical polysome profiles from quiescent cells pretreated with rapamycin are indistinguishable from control cells treated with the vehicle alone (compare Fig. 2*B 1* and *4*): ≈80% of the ribosomes are present as inactive 80S couples. That the latter are 80S ribosomes and not monosomes was demonstrated by their dissociation into 40S and 60S ribosomal subunits after centrifugation through high-salt gradients (ref. 19 and data not shown). By 60 min after serum stimulation a large portion of the 80S ribosome population has been recruited into actively translating polysomes (Fig. 2*B 2* and *5*). However, in cells treated with rapamycin there is a small, but marked, difference in the rate at which 80S ribosomes redistribute to polysomes, as compared with untreated cells. This difference, although small, is still clearly distinguishable at 2 hr (data not shown), as well as 3 hr, after serum stimulation (Fig. 2*B 3* and *6*) and is consistent with the inhibitory effects observed on global protein synthesis. Thus, rapamycin has a small, but significant, inhibitory effect on the increase in the initiation of protein synthesis in Swiss 3T3 cells.

Selective Effects: eEF-1α vs. β-Actin. The inhibitory effect of rapamycin on protein-synthesis initiation did not appear large enough to explain the seemingly greater effects on cell growth observed in Swiss 3T3 cells (ref. 3 and C.R., unpublished data), which prompted the question of whether the translation of specific transcripts was altered. Recently we showed that eEF-1α mRNA is under selective translational control (19). Because S6 phosphorylation has been implicated in differential recognition of specific transcripts (26), we examined the fate of eEF-1α mRNA by S1 solution hybridization after serum stimulation. As a control, the serum-induced transcriptional increase in a transcript of similar size, β-actin, was followed. In quiescent cells, eEF-1α mRNA largely distributes in two populations, monosomes/disomes and stored mRNA-protein particles, whereas most

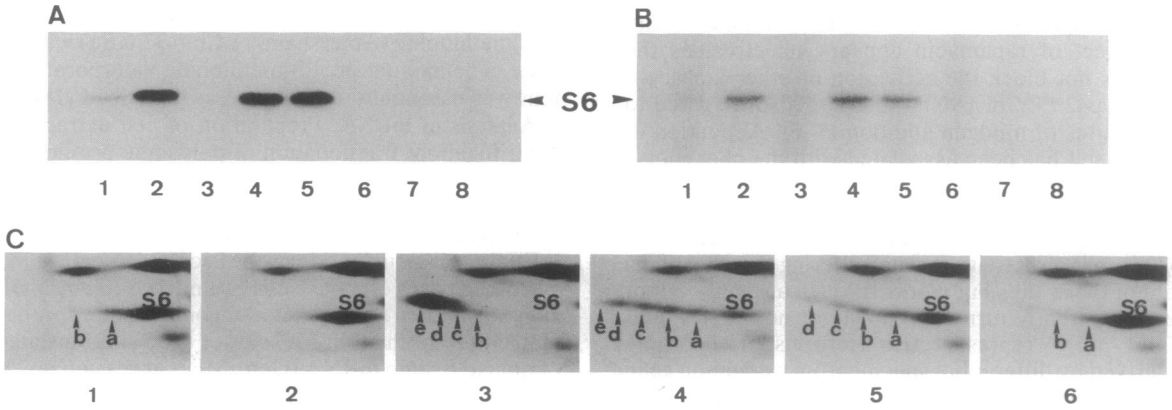

FIG. 1. Effect of rapamycin on $p70^{s6k}/p85^{s6k}$ activity and S6 phosphorylation. In *A* and *B* extracts were prepared from quiescent cultures (lane 1) or cultures treated for 3 hr with serum (lane 2), 3 hr with serum in the presence of rapamycin (lane 3), 3 hr with serum in the presence of vehicle alone (lane 4) or treated with serum for 3 hr and then with vehicle alone for 30 min (lane 5) or rapamycin for 30 min (lane 6), 60 min (lane 7), or 120 min (lane 8). Immunocomplex assays were then done on cell extracts with the M5 antibody (22), which recognizes both isoforms (*A*), or the C3 antibody (9), which recognizes $p85^{s6k}$ (*B*), as described (22). (*C*) Extracts were prepared from quiescent cells (*1*), quiescent cells stimulated with serum for 3 hr with (*2*) and without (*3*) rapamycin or quiescent cells stimulated for 3 hr with serum after which rapamycin was added for 30 min (*4*), 60 min (*5*), or 120 min (*6*). Two-dimensional polyacrylamide gel analysis of ribosomal proteins from individual extracts was done as described (23). Arrowheads indicate derivates a–e, respectively.

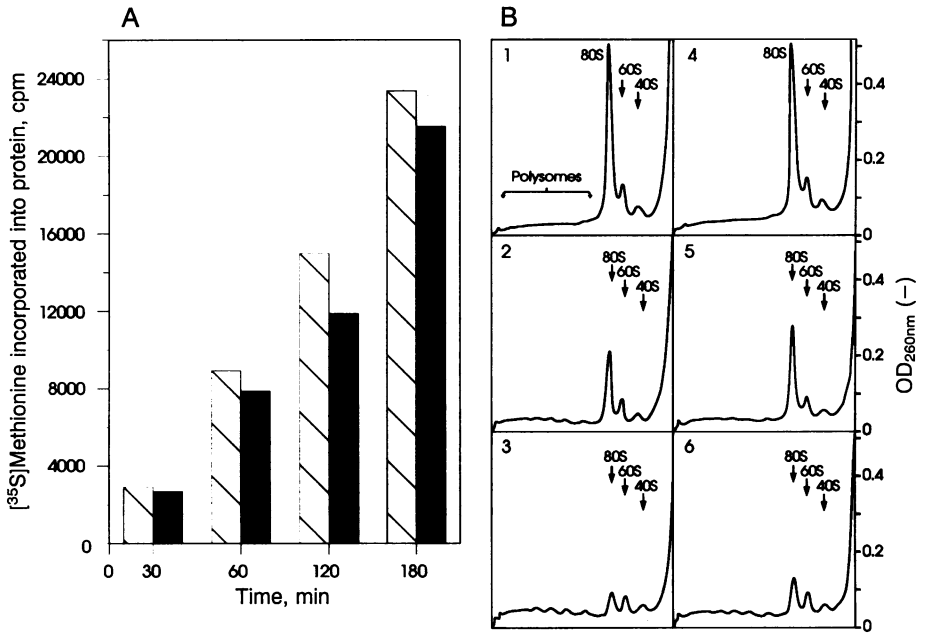

FIG. 2. Protein synthesis and polysome profiles. (*A*) Serum-stimulated cells were labeled with [^{35}S]methionine for the indicated times in the absence (hatched bars) or presence (closed bars) of rapamycin, and the total amount of [^{35}S]methionine incorporated into protein was determined. Protein determinations were made as earlier described (24). (*B*) For analytical polysome profiles, cell extracts were prepared from quiescent cultures (*1* and *4*), 60-min serum-stimulated cultures (*2* and *5*) or 180-min-stimulated cultures (*3* and *6*). Stimulation was without rapamycin (*1*, *2*, and *3*) or with rapamycin (*3*, *4*, and *6*), and extracts were applied to 17.1%–41% exponential sucrose gradients, centrifuged, and analyzed as described (25).

β-actin transcripts are present on polysomes containing six to eight ribosomes (Fig. 3*A* and ref. 19). After serum stimulation, 80S ribosomes are recruited into polysomes, and this response is paralleled by a marked increase in the number of β-actin transcripts (Fig. 3*B*). In contrast to β-actin, the amount of eEF-1α transcripts remains constant during this time, but both populations redistribute to polysomes containing 11 or 12 ribosomes per transcript, a size significantly larger than those occupied by β-actin mRNA (Fig. 3*B*). If cells are stimulated in the presence of rapamycin, the amount of 80S ribosomes recruited into polysomes is reduced (Fig. 3*C*), consistent with the results obtained in Fig. 2*B*. Such treatment has no effect on the distribution of β-actin transcripts nor on most other transcripts, as the mean polysome size remained unaltered. However, under these conditions, the shift of eEF-1α to larger polysomes is severely repressed. Because rapamycin treatment of stimulated cells induced significant S6 dephosphorylation within 1 hr (Fig. 1*C5*), the

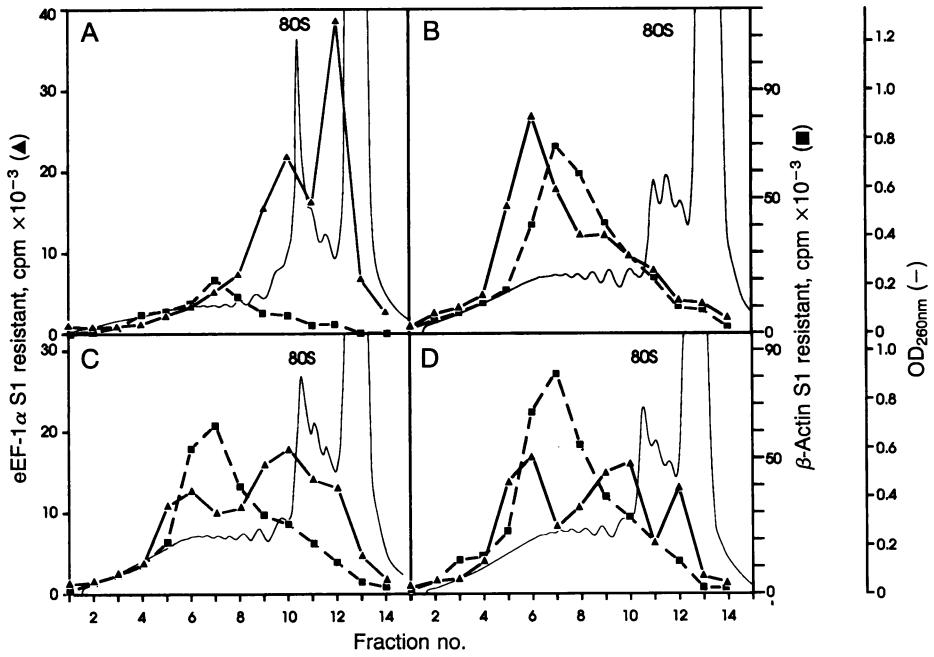

FIG. 3. Polysome distribution of eEF-1α and β-actin mRNA. Cytoplasmic extracts from quiescent cells (*A*), 180-min serum-stimulated cells without rapamycin (*B*), or with rapamycin continuously present (*C*) and 180-min serum-stimulated cells treated with rapamycin for another hour (*D*) were applied to 17.1%–51% linear sucrose gradients, centrifuged, and analyzed by solution hybridization, as described (19). ▲, eEF-1α; ■, β-actin.

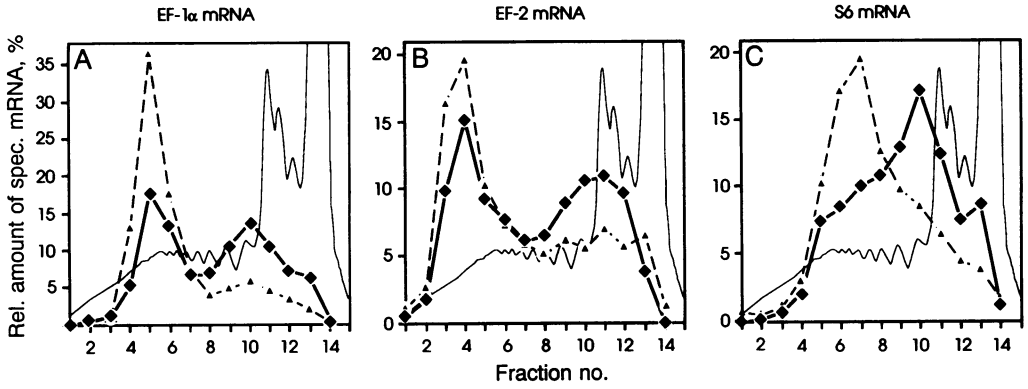

FIG. 4. The effect of rapamycin on the polysome distribution of polypyrimidine-track-containing mRNAs. (*A*) eEF-1α. (*B*) eEF-2. (*C*) Ribosomal protein S6. Cytoplasmic extracts from cells stimulated with serum for 180 min without (▲) or with (◆) 30-min rapamycin pretreatment were applied to 17.1%–51% linear sucrose gradients and after centrifugation analyzed in Northern blots (ref. 19 and text). Blots were then exposed to a PhosphorImager (Molecular Dynamics), and the signals were quantitated by IMAGEQUANT (Molecular Dynamics) and calculated as percentage of total signal for that probe. The thin continuous line depicts the corresponding polysome profile. Rel., relative; spec., specific.

effect of the immunosuppressant on the distribution of the two transcripts was also examined under these conditions. The results show that such treatment leads to a small, but significant, polysome run-off and an increase in 80S ribosomes without affecting the mean polysome size, implying that rapamycin treatment exerts an inhibitory effect on initiation of protein synthesis. Consistent with this finding, a significant portion of eEF-1α mRNA redistributes from large polysomes to monosomes/disomes and stored mRNA–protein particles, whereas β-actin transcripts remain unaffected (compare Fig. 3*B* with 3*D*). Treatment for only 30 min with rapamycin led to a partial redistribution of eEF-1α, whereas treatment for 2 hr gave essentially the same profile as seen after 1 hr (data not shown). Thus, rapamycin treatment appears to selectively suppress the translation of eEF-1α mRNA.

Polypyrimidine Motif. The 5′ untranslated region of human eEF-1α mRNA contains a polypyrimidine tract immediately downstream of its N^7-methylguanosine cap (27). The polypyrimidine-tract sequence is conserved in mouse eEF-1α genomic DNA (28). All vertebrate ribosomal protein mRNAs described to date have such a polypyrimidine tract, which can act as a translational modulator (29). To determine whether the expression of other transcripts containing this sequence are inhibited by rapamycin, the translation of ribosomal protein S6 and elongation factor eEF-2 was examined by Northern blot analysis; the two transcripts behave similarly. In quiescent cells they are largely distributed in monosomes/disomes and mRNA–protein particles (data not shown). After serum stimulation these transcripts shift to larger polysomes (Fig. 4), whereas in the presence of rapamycin this shift is suppressed in both cases (Fig. 4). A similar result is obtained when rapamycin is added to 3-hr serum-stimulated cells for 1 hr (data not shown) or when two other transcripts were examined that contain this motif, ribosomal protein L32 (29) and Q_{23} (19). Thus, rapamycin treatment selectively inhibits the translation of a class of mRNAs that share a common motif that modulates translation, implying this may be the mechanism by which rapamycin inhibits cell growth.

DISCUSSION

Our data show that rapamycin treatment efficiently blocks $p70^{s6k}/p85^{s6k}$ activation and S6 phosphorylation and that a similar net result is achieved when the macrolide is added to cells pretreated with the mitogen. In parallel, such treatment also suppresses the rate of initiation of protein synthesis and, more dramatically, the translation of a class of mRNA transcripts that contain a polypyrimidine tract immediately after their 5′ N^7-methylguanosine cap. It should be noted, though, that the pattern and extent of translational inhibition differ for each transcript containing this motif. Previously we demonstrated that microinjection of $p70^{s6k}/p85^{s6k}$ IgGs into the cytoplasm of quiescent rat-embryo fibroblasts also inhibited the subsequent serum-induced increase in protein synthesis (22). However, the effect of antibody injection was greater than that observed after rapamycin treatment; whether this difference is inherent in the two approaches used should be resolved by studies with mutant forms of the kinase.

Recently we showed that expression of eEF-1α is under selective translational control after mitogenic stimulation of quiescent cells (19). A second protein, previously designated Q_{23} (16, 17), was found to be regulated similarly. These two transcripts contain a polypyrimidine tract immediately 3′ of their *N*-methylguanosine cap, a structure that is present in all sequenced vertebrate ribosomal protein mRNA transcripts and recently shown to confer translational control on expression of this family of mRNAs (29–32). Our results demonstrate that the translation of transcripts which contain this motif are selectively inhibited by rapamycin treatment, whereas the translation of transcripts lacking this motif, such as β-actin as well as β-tubulin and initiation factor eIF-4A (data not shown), are not affected. Preliminary results using stably transfected chimeric constructs that contain the intact polypyrimidine tract or in which a portion of the tract has been replaced by purines indicate that this motif is necessary for rapamycin to exert its inhibitory effect on translation of this mRNA family (H.B.J.J., C.R., Silvian Shama, Oded Meyuhas, and G.T., unpublished data). We also note that the inhibitory effect of rapamycin is not complete (Figs. 3 and 4), implying that other elements may be involved in up-regulating the expression of this mRNA family. To determine whether some of these elements lie in other portions of the 5′ untranslated region, it will be of interest to construct chimeric transcripts and to examine their translation with rapamycin.

If rapamycin selectively inhibits the translation of this family of transcripts, what then is the underlying mode of macrolide action? A protein of M_r 56,000–57,000 from mouse, bovine, and *Xenopus* has recently been shown to bind specifically to the polypyrimidine tract and not to bind to the 5′ untranslated region of mRNAs lacking the tract (32, 33). In neither mouse, bovine, nor *Xenopus* does the binding activity of this factor change as a function of growth or developmental state (32, 33). These results have led both groups to speculate that translational control is modulated by some other factor which interacts with this protein or the polypyrimidine tract.

One obvious candidate would be phosphorylated ribosomal protein S6. As shown here, the kinetics of S6 phosphorylation and dephosphorylation closely parallel the effect of rapamycin on the translation of these mRNAs. Furthermore, the location of S6 in the 40S ribosome, close to the mRNA-binding site, would position it to exert such an effect. Indeed, earlier studies suggested that differentially phosphorylated S6 could recognize specific substrates with unique affinities (26). Biochemical and genetic approaches used to examine the role of other phosphorylated translational components in protein synthesis (34, 38) could be envisaged to test the role of S6.

If it is argued that rapamycin exerts its inhibitory effects on cell growth through the $p70^{s6k}/p85^{s6k}$, S6 phosphorylation, and the translation of polypyrimidine tract-containing transcripts, the question arises why certain cell types are more affected than others. One explanation is that those cells severely inhibited in growth are more dependent on this family of mRNAs or that their translation is more dramatically altered in these cells. As the proteins thus far identified that contain this motif in their cognate mRNAs are essential gene products, their inhibition should significantly affect cell growth. We also note that such a mechanism for rapamycin may explain some of the delayed G_1 late effects recently seen on the activities of the $p33^{cdk2}$ and $p34^{cdc2}$ kinases (35–37), as pointed out by Morice *et al.* (37). Indeed, $p70^{s6k}/p85^{s6k}$ activity and S6 phosphorylation, unlike many early mitogenic responses, remain high throughout G_1 phase (22). Looking at the translation of polypyrimidine-tract mRNA in cells that have a proliferation rate highly sensitive to rapamycin will be of obvious interest.

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