In mammalian cells, the p70 and p85 isoforms of the 70-kDa ribosomal protein S6 kinase (p70S6K) are both rapidly activated upon stimulation of cells with virtually all mitogenic factors (31, 46, 60). The two isoforms are identical except that p85 has an amino-terminal 23-amino-acid extension containing a nucleolar localization signal. The two isoforms are derived from the same transcript by alternative translation initiation start sites (50a). p70S6K is activated by a complex pattern of phosphorylation on several sites by various upstream kinases (48, 60). The first event is phosphorylation of (Ser/Thr)-Pro sites Ser411, Ser418, Thr421, and Ser424 in the carboxy-terminal autoinhibitory domain, facilitating phosphorylation of Thr389 and Ser404 in the linker region, which in turn leads to disruption of the interaction between the carboxy and amino termini of the protein. Finally, phosphorylation of Thr229 leads to full activation of p70S6K (32). The kinase responsible for Thr229 phosphorylation is the constitutively active 3-phosphoinositide-dependent protein kinase 1 (1a, 49a), whereas the kinases involved in the previous phosphorylation events, also required for activation, are not clearly identified. However, phosphorylation of Thr389 in mammalian cells is dependent on the kinase activity of target of rapamycin (TOR), also termed FRAP, RAFT, or RAPT (9, 48, 60). The macrodil antibiotic rapamycin is a potent inhibitor of the p70S6K pathway. It forms a complex with FKBP12, which specifically blocks activity of mammalian TOR, thereby leading to rapid deactivation of p70S6K (1, 9, 16, 31, 58). Rapamycin has been shown to down-regulate the translation of mRNAs containing a 5′-terminal oligopyrimidine tract (5′-TOP), which include those for ribosomal proteins and other proteins of the translational machinery (4, 28, 29, 41, 59). All known mRNAs for vertebrate ribosomal proteins and protein synthesis elongation factors contain a 5′-TOP, and their translation is regulated in response to mitogens (2–5, 30, 31, 41) via p70S6K activity (28).

The downstream target of p70S6K is ribosomal protein S6, which is present in a single copy per 40S subunit. S6 becomes rapidly phosphorylated on five serine residues in its carboxy-terminal region upon stimulation of cells with growth factors. Moreover, phosphorylation occurs in an ordered manner in vivo and in vitro (6, 63). S6 phosphorylation is closely correlated with increased rates of protein synthesis when quiescent cells reenter the cell cycle (17, 58). Conversely, a decrease in protein synthesis is paralleled by lower S6 phosphorylation (58). The concept that the function of p70S6K is linked to regulation of protein synthesis is also suggested by studies in marine embryonic stem cells with a disrupted p70S6K gene (32).

Other studies have suggested that p70S6K is also linked to pathways controlling cell cycle progression. In particular, microinjection of neutralizing p70S6K antibodies into mammalian cells inhibits G1 progression (35). Moreover, mice deficient for p70S6K are significantly smaller, an effect which is most dramatic during embryogenesis. Unexpectedly, mouse embryo fibroblasts derived from p70S6K-deficient mice were as sensitive to rapamycin as mouse embryo fibroblasts derived from wild-type animals, and there was no effect on the S6 phosphorylation response (57). These studies led to the identification of a new highly homologous S6 kinase which is rapamycin sensitive.
and whose transcript is up-regulated in all tissues examined (57). The importance of p70S6K in both protein synthesis and cell cycle progression awaits studies in mice deficient for both S6 kinase genes.

Xenopus oocytes are an interesting system for the study of S6 phosphorylation because of the amplification of ribosomal genes and ribosomes during oogenesis. It has been estimated that in oocytes only 1% of the ribosomes are present on polysomes, with the remainder being gradually utilized after fertilization (64). During progesterone-induced oocyte maturation in X. laevis, S6 phosphorylation by PDK1 on Ser^47 in the total ribosome population changes dramatically. It is low in resting oocytes, is increased greatly when 50% of the oocytes have undergone germinal vesicle breakdown (GVBD), and is maximal in unfertilized eggs (44). In parallel, overall protein synthesis is up-regulated by a factor of approximately 2 (51). Biochemical and molecular cloning studies have indicated the protein kinase responsible for S6 phosphorylation during maturation is p90^Bsk (19–21). Like p70S6K, Rsk phosphorylates all five sites in S6 in an ordered fashion. Despite the general increase in protein synthesis and S6 phosphorylation during oocyte maturation, production of ribosomal proteins ceases (27), suggesting that translation of ribosomal protein mRNAs (rp-mRNAs) that contain the 5'-TOP is uncoupled from that of non-5'-TOP mRNAs during oocyte maturation and may be regulated by different mechanisms (2–5). After fertilization, translation of S3, L17, and L31 begins, and L5 synthesis is evident from stage 7 onward (49). In both oocytes and embryos, total translational capacity is constant such that new mRNAs compete for translation with existing mRNAs (37).

The p70S6K has been reported to become rapidly deactivated after induction of Xenopus oocyte maturation, suggesting a role in resting oocytes that is terminated in the initial phase of oocyte maturation (36). p70S6K function has not previously been investigated in embryos. Therefore, we cloned a full-length Xenopus homolog of p70S6K and investigated the function of this enzyme during oocyte maturation and early development in X. laevis. p70S6K was not responsible for the up-regulation of S6 phosphorylation during maturation. Indeed, administration of rapamycin accelerated oocyte maturation that was correlated with reduced translation of mRNAs with a 5'-TOP region and enhanced translation of mRNAs. In embryos, p70S6K was rapidly activated after fertilization and may contribute to the enhanced translation of several ribosomal proteins after fertilization.

**MATERIALS AND METHODS**

Cloning of X. laevis p70S6K cDNA. A cDNA library generated by oligo-dT priming of RNA from defolliculated X. laevis oocytes was obtained from D. Melton (50). Screening of 4X10^7 PFU by hybridization with probes corresponding to the 251 bp of the 5' coding sequence of rat p70S6K cDNA and to 665 bp of a partial X. laevis cDNA clone (36), respectively, revealed 20 clones containing identical 1.7-kb inserts. Two phage inserts were subcloned in pBluescript and analyzed by sequencing of both strands, using a Sequenase 2.0 sequencing kit (U.S. Biotechnology, Lake Placid, N.Y.) with oligonucleotide primers from the T3 and T7 sequences in the vector polylinker.

Oocytes, eggs, and embryos. Female X. laevis eggs were injected with 75 µg of pregnant mare’s serum gonadotropin (PMSG) 2 to 7 days prior to dissection of the ovary and manual isolation of oocytes. Isolated oocytes were incubated in 1X modified Barth’s solution [88 mM NaCl, 1 mM KCl, 0.41 mM CaCl_2, 0.33 mM NaN_2SO_4, 0.82 mM MgSO_4, 2.4 mM NaHCO_3, 10 mM HEPES (pH 7.4)], and maturation was induced by addition of progesterone as indicated in the figure legends. Rapamycin (Sigma, St. Louis, Mo.) dissolved in dimethyl sulfoxide, was added at a final concentration of 2 µg/ml to 2 h prior to induction of maturation. Controls were exposed to dimethyl sulfoxide alone. When eggs or embryos were needed, frogs were injected with 550 µl of human chorionic gonadotropin to induce egg laying 12 to 14 h later. To obtain activated eggs, freshly laid eggs were defolliculated with 2% cycloheximide (100 mM MMR; 100 mM NaCl, 2 mM KCl, 1 mM CaCl_2, 1 mM MgCl_2, 5 mM HEPES [pH 7.4]), treated with the calcium ionophore A23187 (Calbiochem, La Jolla, Calif.) at a final concentration of 5 µg/ml in 1X MMR for 1.5 to 2 min, and rinsed 8 to 10 times with 0.2X MMR. Preincubation with rapamycin (2 µg/ml in 1X MMR) was performed for 15 to 30 min before addition of the calcium ionophore. Embryos were obtained by in vitro fertilization of frogs that had been likened in 0.1X MMR as described previously (26). Embryos were staged as described by Nieuwkoop and Faber (45). Oocytes, eggs, and embryos were frozen in dry ice at the desired time or stage.

**Injection of mRNA and DNA.** Constructs encoding Myc-tagged active and inactive mutants of rat p70S6K were produced by PCR amplification of plasmids pRK5-myc-p70S6K-D3E, pRK5-myc-p70S6K-D3E (42, 48), using the primers 5'-CTCAGCTGCAGGAGTGTGATACGGAGG-3’ and 5’-GCGTCCTCAAGATATTCATCTACACGAC-3’, PCR products were digested with T7 Easy vector (Promega, Madison, Wis.); the resulting plasmids were digested with EcoRI and the fragment was ligated into EcoRIdigested pC22-MT (53, 62). Capped mRNA was produced in vitro by using a Message Machine kit (Ambion, Austin, Tex.) after linearization of the plasmid with NotI. Oocytes were injected with 20 ng of mRNA and incubated for 2 h prior to induction of maturation by progesterone. The 5'-flanking region of the hamster elongation factor 2 (EF2) gene (with [-727 to +47] or without [-727 to +1] transcription initiation site) a 5'-TOP) was inserted upstream of luciferase cDNA in the pGL2-Enhancer vector (Promega) to obtain the EF2+TOP or EF2- TOP luciferase reporter. The hamster EF2 gene has a typical 5'-TOP sequence from its transcription initiation site (+1 CTTCCCTAGCCCAAGACCCGGCCACG TGCACCCAGGCTCCTTTC-+47) (43). Initiation of transcription of the chimeric mRNAs at the native EF2 transcription site was confirmed by S1 mapping in a mammalian cell plasmid. Plasmid DNA (1.5 ng) encoding luciferase reporters with or without a 5'-TOP, driven by the genomic promoter of hamster EF2, was injected into the oocyte germinal vesicle. After incubation at room temperature for 10 h, oocytes were frozen in dry ice.

The SP6-EF2+TOP reporter construct, which was used for production of RNA, was made by PCR amplification of EF2+TOP by using the primers GAT TTTAGGATCCATATGCTCAGCCGAGCAC and CTCAGCTTCGCCATCTTTTACGAGTC and anti-p70 S6K) or with rabbit anti-Cdc25A antibodies or onto 12.5% Anderson gels for immunoblotting with rabbit anti-p70 S6K antibodies, after transfer to nitrocellulose membranes by using a semi-dry blotting technique (Pharmacia-LKB, Piscataway, N.J.). Membranes were blocked with 10% nonfat dry milk in phosphate-buffered saline–0.05% Tween 20 and probed with antibodies in phosphate-buffered saline–0.05% Tween 20.

**Gel electrophoresis and Western blotting.** Oocytes, eggs, or embryos were homogenized in extraction buffer (50 mM Tris [pH 7.4], 80 µM β-glycerophosphate, 20 mM EDTA, 20 mM NaF, 0.1 mM sodium vanadate, 1 mM diithiothreitol [DTT], 0.3 µM microcystin, 0.3 mM phenylmethlysulfonyl fluoride, leupeptin [10 µg/ml], pepstatin [10 µg/ml], chymostatin [10 µg/ml] and centrifuged at 5 min at 4°C. The cytosolic phase equivalent to one oocyte, egg, or embryo was loaded on Laemmli sodium dodecyl sulfate (SDS)–10% polyacrylamide gels for electrophoresis with rabbit anti-Mos (Santa Cruz Biotechnology, Santa Cruz, Calif.) or rabbit anti-Cdc25A antibodies or onto 12.5% Anderson gels for immunoblotting with rabbit anti-p70S6K antibodies, after transfer to nitrocellulose membranes by using a semi-dry blotting technique (Pharmacia-LKB, Piscataway, N.J.). Membranes were blocked with 10% nonfat dry milk in phosphate-buffered saline–0.05% Tween 20 and probed with antibodies in phosphate-buffered saline–0.05% Tween 20.
tinely, extract corresponding to 0.05 or 0.5 oocyte was assayed for luciferase activity by the injection of 100 μl of luciferase substrate (Promega) into a Mono Light luminometer (Analytical Luminescence Laboratory, Ann Arbor, Mich.) according to the manufacturer’s protocol. For detection of β-galactosidase activity, 10 μl of extract corresponding to 0.5 oocyte was incubated with 70 μl of β-galactosidase reaction buffer (Tropix) for 2 h at room temperature before injection of 100 μl of light emission accelerator into the luminometer.

Nucleotide sequence accession number. The sequence shown in Fig. 1A has been deposited in the GenBank under accession no. AJ131521.

RESULTS

Cloning of X. laevis p70S6K cDNA. Earlier studies led to the identification of a PCR product encoding a maternal form of p70S6K from X. laevis (36). Hybridization screening of an X. laevis cDNA library with rat and Xenopus probes identified multiple clones with a 1.7-kb insert. The insert sequence of 1,717 nucleotides contains a large open reading frame. The first ATG codon, nucleotides 97 to 99, is surrounded by a long 5′-untranslated region, and the following 1,503 nucleotides encode an amino acid sequence with 93% identity to the sequence of mammalian p70S6K (Fig. 1). Longer clones containing sequences homologous to the p85S6K amino terminus were not found; however, no stop codon was found in frame in the 5′-untranslated sequence preceding the ATG translation initiation codon. In addition, none of the other 20 phases isolated from the cDNA library contained a longer 5′-untranslated sequence.

p70S6K activity during oocyte maturation and embryonic development. In the earlier study described above, Lane et al. (36) showed that the activity of p70S6K in oocytes declined after induction of maturation by progesterone. The high sequence identity of Xp70S6K with the mammalian enzyme (Fig. 1) plus conservation of all the phosphorylation sites and regulatory motifs present in the mammalian enzyme supports the use of reagents based on mammalian p70S6K for study of the Xenopus enzyme. In the experiments reported here, we determined the activity profile of p70S6K during oocyte maturation by assaying the kinase activity of immunoprecipitated p70S6K, using 40S ribosomal subunits as the substrate. p70S6K activity was high in resting oocytes, decreased 6- to 10-fold within the first 2 h after induction of maturation by progesterone, and stayed low until oocytes reached germinal vesicle breakdown (GVBD) (Fig. 2). Similar results were obtained with oocytes from PMSG-primed and unprimed frogs, and kinase activity in resting oocytes was in the same range in both primed and unprimed oocytes. Usually no increased activity was evident after progesterone treatment, but in one experiment a 15% increase in p70S6K activity was seen upon stimulation of oocytes with progesterone (Fig. 2A). Western blotting confirmed that electrophoretic shifts mirror the p70S6K activity changes observed with immunocomplex kinase assays (Fig. 2B). The anti-p70S6K antibody recognized two clusters of bands of equal intensity at ~70 and ~85 kDa, representing the p70 and p85 isoforms. These blots indicate that the p85 isoform described in other species is also present in X. laevis, and both isoforms undergo changes in activity together during maturation. In immunoblots, both isoforms could be blocked by preincubation of the antibody with the immunogenic peptide (data not shown). The bands in each cluster have previously been shown to differ in phosphorylation state (48). The samples with the highest enzyme activity showed multiple bands for each isoform (lanes 1 and 7). With the gradual decrease of activity after induction of maturation, the most retarded isoforms disappeared and the abundance of the isoforms with higher mobility increased (lanes 2 to 6 and 8 to 12). Thus, the antibody recognized both isoforms on the Western blot, and it also precipitated both forms from extracts (data not shown). Therefore, in kinase assays both the p70 and p85 isoforms appear to contribute to total S6 kinase activity.

After fertilization of X. laevis eggs, all new protein synthesis is translationally controlled prior to the midblastula transition (MBT) (18). Therefore we also investigated the activity of p70S6K during early embryonic development (Fig. 2C). After fertilization, p70S6K activity increased about 30-fold during the first cell cycle and then decreased over the next two to three cell cycles. Activity increased again two- to threefold at stage 5, shortly before the MBT, and a third peak was observed during gastrulation at stage 12. The results of the kinase assays were confirmed by changes in the abundance of slower-migrating bands of both isoforms on Western blots (Fig. 2D).

The first cell cycle is the only pre-MBT cell cycle to contain a G phase. Therefore, the detailed kinetics of the increase of p70 activity in the first cell cycle after fertilization was studied. Since embryos require a 30-min dejellling procedure before extracts can be made, we measured p70S6K activity in dejellled unfertilized eggs activated with the calcium ionophore A23187, which mimics the events of fertilization. p70S6K activity increased to a high level within the first 30 min after ionophore treatment, with significant activity by 20 min after treatment (Fig. 3A). The deadenylation of mos mRNA after fertilization and the degradation of Mos protein after activation of eggs paralleled increases in p70S6K activity (Fig. 3B). These results indicate that in X. laevis eggs, p70S6K can be activated in the absence of growth factors solely by elevation of cellular calcium levels, as has been shown in other systems (14, 25).

Rapamycin accelerates oocyte maturation and decreases the threshold level of progesterone required for maturation. The activity profile of p70S6K after progesterone treatment suggests that an even earlier down-regulation of its activity might play a role in oocyte maturation. Rapamycin has been shown to be a potent inhibitor of the p70S6K pathway in different systems, specifically inhibiting TOR, a mediator of p70S6K function (1, 16). Thus, to investigate the function of p70S6K during oocyte maturation and embryogenesis, rapamycin was used to block activation of the enzyme in oocytes and embryos. Incubation of oocytes in rapamycin (2 μg/ml) decreased the activity of p70S6K to the background level (Fig. 4A). In embryos treated with rapamycin after fertilization, p70S6K activity was also reduced to the background level. At 30 min after fertilization, corresponding to 10 min after addition of rapamycin, activity was lower than in control embryos (Fig. 4B). At all later time points, 45 to 105 min postfertilization, p70S6K activity was undetectable. This indicates that it takes between 10 and 30 min to fully inhibit p70S6K activity by incubation of oocytes and embryos in rapamycin.

Surprisingly, oocytes treated with rapamycin underwent GVBD faster than untreated oocytes (Fig. 5). Fifty percent GVBD (GVBD_{50}) occurred between 1 and 2 h earlier at the minimum concentration of progesterone that led to 100% GVBD (Fig. 5A), which is different for oocytes from different frogs. This effect of rapamycin could not be detected in oocytes treated with a high dose of progesterone, indicating that high concentrations of progesterone can override the molecular effects of rapamycin. Although rapamycin alone, without addition of progesterone, was not able to induce oocyte maturation, it caused a higher percentage of GVBD in treated oocytes at suboptimal concentrations of progesterone that cause less than 100% GVBD (Fig. 5B). Therefore, rapamycin not only is able to accelerate maturation but also increases the sensitivity of oocytes to progesterone, suggesting that the progesterone-dependent down-regulation of p70S6K activity is important for normal maturation kinetics.
Rapamycin does not inhibit p70S6K directly but instead inhibits the upstream kinase TOR, which leads to rapid deactivation of p70S6K (1, 16). To exclude the possibility that the effect of rapamycin on oocyte maturation was due to inhibition of another downstream target of TOR, such as 4E-BP1/PHAS I (9, 38, 60), oocytes were injected with 20 ng of in vitro-transcribed mRNA encoding a constitutively active and rapamycin-insensitive mutant of rat p70S6K (p70S6KD3E-E389). The protein level of the expressed kinase was about 5- to 10-fold above that of the endogenous p70S6K (data not shown).
extracts from oocytes injected with the constitutively active p70S6K, total p70S6K activity was about 30-fold higher than in uninjected samples at GVBD (Fig. 6A, lanes 1 and 2). Kinase activity in oocytes injected with an inactive mutant of rat p70S6K (p70S6KQ100) was the same as in uninjected control oocytes (Fig. 6A, lanes 1 and 3). Importantly, at a subthreshold concentration of progesterone, injection of the active form of p70S6K reversed the accelerating effect of rapamycin, whereas oocytes injected with the inactive form of p70S6K behaved like uninjected oocytes incubated with rapamycin (Fig. 6B). This result suggests that the acceleration of oocyte maturation by rapamycin is indeed mediated by specific blocking of the p70S6K pathway and not by an alternative pathway that is also blocked by rapamycin.

**Rapamycin does not block increased S6 phosphorylation during maturation.** If inhibiting p70S6K accelerates maturation, one might have predicted that expression of a constitutively active p70S6K would not only reverse the effects of rapamycin on acceleration of GVBD but also retard the kinetics of GVBD with low-dose progesterone. As shown in Fig. 6, elevated p70S6K activity does not retard maturation. A possible explanation for this result is that the normal function of p70S6K to phosphorylate S6 is being performed in progesterone-treated oocytes by p90Rsk. In support of this idea, p90Rsk was originally purified as the only S6 kinase activity present in fully mature eggs (19–22), and p70S6K activity is undetectable by GVBD (Fig. 2 and reference 36). Moreover, p90Rsk phosphorylates all five sites in S6 in the same ordered fashion as observed with p70S6K (63). Thus, it is likely that in p70S6K-injected oocytes at GVBD, S6 phosphorylation is already maximal due to the activity of p90Rsk. To test this hypothesis further, we determined the levels of S6 phosphorylation in the presence and absence of rapamycin (44). The results show that S6 phosphorylation was greater in progesterone-treated than in untreated oocytes, with the majority of the protein migrating in derivatives b and c, containing 2 and 3 mol, respectively, of phosphate (Fig. 7A and B, respectively), as reported previously (44). In the presence of rapamycin the progesterone

![FIG. 2. Activity of p70S6K during oocyte maturation and early embryogenesis.](http://mcb.asm.org/)

![FIG. 3. p70S6K activity increases within 30 min after activation of eggs.](http://mcb.asm.org/)
response was largely unaffected, although slightly less phosphorylated S6 derivative d was evident (compare Fig. 7B and C). The insensitivity of S6 phosphorylation to rapamycin (Fig. 7B) supports the identification of p90Rsk as the enzyme responsible for increased S6 phosphorylation in response to progesterone treatment. Recently, a second immunologically distinct form of p70S6K, termed p70S6K2, was identified in p70S6K1-deficient mice (57). However, this enzyme is also rapamycin sensitive and therefore unlikely to account for S6 phosphorylation in oocytes when p70S6K1 is down-regulated (Fig. 2).

Despite the fact that p90Rsk accounts for S6 phosphorylation during maturation, it cannot be excluded that p70S6K regulates translation of mRNAs on the minor (1%) fraction of ribosomes that are on polysomes in resting oocytes, since the constitutively active p70S6K overcomes rapamycin effects that are evident before Rsk activation (Fig. 6).

**FIG. 4.** Rapamycin inhibits the activity of p70S6K in oocytes and embryos. (A) Oocytes were pretreated with rapamycin (2 μg/ml) for 1 to 2 h, and maturation was induced by addition of progesterone (10 μg/ml). Samples were collected, and S6 kinase activity was measured from immunoprecipitated p70S6K from an extract corresponding to one oocyte per sample. Results are expressed relative to p70S6K activity in stage VI oocytes. (B) Freshly laid eggs were fertilized in vitro, dejellied, and treated with rapamycin (2 μg/ml) 20 min after fertilization. Extracts were prepared at the indicated times, and kinase activity was measured from immunoprecipitated p70S6K.

**FIG. 5.** Rapamycin accelerates GVBD and decreases the threshold concentration of progesterone required for maturation. (A) Oocytes were pretreated with rapamycin (2 μg/ml) for 2 h before induction of maturation with a threshold level of progesterone. The percentage of GVBD was scored by occurrence of a well-defined white spot in the animal pole indicative of GVBD. At the lowest concentration of progesterone that leads to 100% GVBD in untreated oocytes (60 ng/ml), GVBD occurred ~1 h earlier in rapamycin-treated oocytes than in controls. (B) At a subthreshold concentration of progesterone (40 ng/ml), at which only 30% of oocytes underwent GVBD, the final percentage of GVBD was increased ~2-fold by rapamycin treatment.

**FIG. 6.** Constitutively active p70S6K reverses the effect of rapamycin. (A) Oocytes were pretreated with rapamycin (2 μg/ml) for 1 to 2 h and then micro-injected with 20 ng of mRNA encoding either p70S6K D3E-E389 (active form) or p70S6K Q100 (kinase-dead form). After incubation for 2 h at room temperature, S6 kinase activity of immunoprecipitated p70S6K was measured in extracts of resting or matured oocytes. Kinase activity was about 30-fold higher at GVBD in oocytes injected with the active form of p70S6K than in control GVBD oocytes. (B) Oocytes were treated with rapamycin (rap.), and some were then injected with the constitutively active (p70S6K D3E-E389) or inactive (p70S6K Q100) form of rat p70S6K. Maturation induced with progesterone (40 ng/ml) occurred a lower percentage in oocytes injected with the active isoform of p70S6K in the presence of rapamycin, whereas inactive p70S6K did not affect the kinetics of maturation.
to 40% higher luciferase activity in extracts from oocytes injected with the construct that did not contain a 5′-TOP (Fig. 9A). This result supports the hypothesis that rapamycin increases translational capacity for non-5′-TOP mRNAs by inhibiting translation of mRNAs with a 5′-TOP region. These results were confirmed in studies using direct injection of mRNAs. The 5′-TOP construct described above was transcribed in vitro and injected into oocytes in the presence and absence of rapamycin. Rapamycin treatment caused a 30 to 40% decrease in translation (Fig. 9B), similar to the level of inhibition after e clone injection. To evaluate whether this decrease led to a commensurate increase in available translational capacity for other mRNAs, we used injection of an mRNA with an internal IRES, which should be independent of many complex 5′ UTR controls. Indeed, recent studies have shown that in virus-infected cells treated with rapamycin, the viral IRES-containing transcripts are more efficiently translated (8). As shown in Fig. 9B, translation of an IRES mRNA in oocytes was increased nearly 40% by rapamycin treatment.

**DISCUSSION**

Fully grown stage VI oocytes are arrested in prophase of meiosis I, which corresponds to late G2 phase in the cell cycle. During maturation a hormonal stimulus releases the oocytes from their arrest, inducing completion of meiosis I and progression to metaphase of meiosis II, where they arrest again awaiting fertilization (24, 40, 54). p70S6K activity is present in unmatured stage VI oocytes (reference 36 and Fig. 2A, 2B, and 4A) and in earlier stages (data not shown). Upon induction of maturation by progesterone, activity of p70S6K decreases, suggesting that low kinase activity facilitates progression through the cell cycle in the oocyte. This notion is supported by the...
observation that incubation of oocytes in rapamycin, leading to a complete loss of p70S6K activity, accelerates oocyte maturation and increases sensitivity to progesterone (Fig. 4 to 6).

During oogenesis, each oocyte produces a huge stockpile of 10^{12} ribosomes that supports embryonic development until the swimming tadpole stage (2-5). Recruitment of rp-mRNAs onto polysomes, a measure of translational activity, increases throughout oogenesis, reaching its maximum in stage VI oocytes (7, 10). This elevated accumulation of ribosomal proteins is due largely to the preferential translation of rp-mRNAs in mid to late stages of oogenesis (15). At that time, translation of rp-mRNAs comprises ~20% of total protein synthesis, and ribosomal proteins are the major class of protein being produced. p70S6K activity is present during this period (data not shown) and decreases (Fig. 2) concomitantly with the cessation of ribosomal protein synthesis during oocyte maturation (27). This finding suggests that decreased p70S6K activity may be responsible for the down-regulation of translation of rp-mRNAs, which contain 5’-TOP regions. In our experiments, synthesis of Mos protein, whose mRNA does not contain a 5’-TOP, starts earlier and reaches a higher amount in rapamycin-treated oocytes (Fig. 8A), resulting in accelerated GVBD (Fig. 5). Also, an IRES reporter construct without a 5’-TOP was translated more efficiently in rapamycin-treated oocytes, whereas expression of a construct containing a 5’-TOP was decreased (Fig. 9). This result suggests a model in which preferential translation of rp-mRNAs with 5’-TOP regions occurs in oocytes when p70S6K activity is high. A reduction of p70S6K activity leads to a decrease in translation of rp-mRNAs and the release of translational capacity for mRNAs without a 5’-TOP region, like Mos, which are required for oocyte maturation.

p70S6K activity changes may also affect protein synthesis after fertilization. In the embryo, translation of ribosomal proteins S3, L17, and L31 starts from stage 1 onward, and protein L5 begins to be synthesized around stage 7 (49), corresponding to one of the peaks of activity of p70S6K (Fig. 2C). It is possible that the high p70S6K activity in early embryos contributes to specific translation of these selected rp-mRNAs.

Phosphorylation of ribosomal protein S6 correlates with translation of 5’-TOP RNA, as shown for EF1α in cultured cells after mitogenic stimulation (30). This finding suggests that in cultured cells the rapamycin effects on translation are mediated via p70S6K-dependent S6 phosphorylation. In mitogen-treated cells, the highly phosphorylated derivatives of S6 are selectively found in polysomes (61). In contrast, in oocytes despite increased S6 phosphorylation prior to GVBD (reference 44 and Fig. 7), only 1% of the ribosomes are in polysomes. Moreover, the kinase responsible for this increased rapamycin-insensitive S6 phosphorylation does not appear to be p70S6K, whose activity is undetectable at GVBD, but rather p90Rsk (19, 20) (Fig. 7). An analogous situation occurs after fertilization, when p90Rsk activity and S6 phosphorylation rapidly decrease (reference 19 and unpublished data) despite an increase in p70S6K activity (Fig. 2). Although there is a two-fold increase in total protein synthesis and maximal S6 phosphorylation at GVBD, ribosomal protein 5’-TOP mRNAs are no longer being translated. Indeed, since the p90Rsk-dependent S6 phosphorylation in this system occurs long after p70S6K is inactivated (Fig. 2), it is possible that the minor fraction of ribosomal 40S subunits on polysomes is phosphorylated by p70S6K before and shortly after induction of maturation with progesterone. Similarly, although most S6 is dephosphorylated after fertilization in concert with deactivation of p90Rsk (19, 44), it cannot be excluded that a minor fraction of S6 is phosphorylated by p70S6K after fertilization. Alternatively, these results could indicate that other targets of p70S6K besides S6, such as trans-acting factors (11, 39), account for rapamycin effects on 5’-TOP mRNA translation.

The fact that progesterone down-regulates p70S6K activity and rapamycin affects the progesterone response implies that the rapamycin-sensitive pathway is under hormonal control in oocytes. Although it is evident that p70S6K deactivation in progesterone-treated oocytes is due to dephosphorylation (Fig. 2), at present we cannot distinguish between inhibition of upstream kinases such as mammalian TOR or activation of phosphatases. Since the effect of rapamycin could be observed only at threshold levels of progesterone, it is likely that other mechanisms activated by progesterone also contribute to the switch to preferential translation of non-5’-TOP RNAs during oocyte maturation. These might include changes in both the 3’ and 5’ UTRs of mRNAs such as mos that occur following progesterone treatment (34). Translation of such mRNAs, however, is still constrained by the need for additional translational...
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vol. 19, 1999

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