We show here using synchronized Swiss mouse 3T3 fibroblasts that p70 S6 kinase (p70\(^{S6k}\)) and mitogen-activated protein kinases (p42\(^{mapk}\)/p44\(^{mapk}\)) are not only activated at the G\(_0\)/G\(_1\) boundary, but also in cells progressing from M into G\(_1\). p70\(^{S6k}\) activity increases 20-fold in G\(_0\) cells released from G\(_0\). Throughout G\(_1\), S, and G\(_2\) it decreases constantly, so that during M phase low kinase activity is measured. The kinase is reactivated 10-fold when cells released from a nocodazole-induced metaphase block enter G\(_1\) of the next cell cycle. p42\(^{mapk}\)/p44\(^{mapk}\) in G\(_0\) cells are activated transiently early in G\(_1\) and are reactivated late in mitosis after nocodazole release. p70\(^{S6k}\) activity is dependent on permanent signaling from growth factors at all stages of the cell cycle. Immunofluorescence studies showed that p70\(^{S6k}\) and its isoform p85\(^{S6k}\) become concentrated in localized spots in the nucleus at certain stages in the cell cycle. Cell cycle-dependent changes in p70\(^{S6k}\) activity are associated with alterations in the phosphorylation state of the protein. However, examination of the regulation of a p70\(^{S6k}\)-encoded ribosomal S6 kinase (S6k\(^{p90}\)) and the rsk\(_1\)-encoded ribosomal S6 kinase (S6k\(^{rsk}1\)) showed that the phosphorylation sites control the activity of the enzyme during the cell cycle.

Signaling pathways that operate through tightly controlled protein phosphorylation cascades transduce extracellular signals to various intracellular targets. Some of these targets regulate the transcriptional and translational machinery to ensure proper cell cycle progression, cell growth, or differentiation. Phosphorylation of the S6 protein of 40 S ribosomal subunits is a highly conserved response of animal cells to treatment with growth factors, steroid hormones, phorbol esters, and oncogenes (1). Inhibition of S6 phosphorylation by exposure of cells to the immunosuppressant rapamycin selectively suppresses the translation of certain mRNAs that contain a polyuridine tract at the 5\(\prime\) end (2). These mRNAs encode ribosomal proteins and protein synthesis elongation factors, whose production is required for efficient transit through the G\(_1\) phase of the cell cycle.

Two families of mitogen-stimulated S6 kinases have been identified: the rsk\(_1\)-encoded M\(_1\), 85,000-92,000 S6 kinases (Ref. 3, referred to as p90\(^{S6k}\)) and the M\(_2\), 70,000 and 85,000 S6 kinases (Ref. 4 and 5, referred to as p70\(^{S6k}\) and p85\(^{S6k}\)). A variety of evidence indicates that p90\(^{S6k}\) and p70\(^{S6k}\)/p85\(^{S6k}\) lie on different signaling pathways (6–10). Unlike p70\(^{S6k}\) (8), p90\(^{S6k}\) is phosphorylated and activated by the erk-encoded M, 42,000 and 44,000 mitogen-activated protein (MAP) kinases (Ref. 6, referred to as p42\(^{mapk}\) and p44\(^{mapk}\)) in response to signals transmitted through p21\(^{ras}\), p74\(^{raf}\), and p42\(^{mapk}\) (11). Once activated, p90\(^{S6k}\) and p42\(^{mapk}\)/p44\(^{mapk}\) can be translocated to the nucleus (12, 13), where they are thought to phosphorylate nuclear transcription factors, thus promoting the transcription of genes required for the growth response.

p70\(^{S6k}\) is the physiological S6 kinase activity in mammalian cells (14). p85\(^{S6k}\) is a minor species that is identical to p70\(^{S6k}\) except for the presence of a 23-amino acid extension at the amino terminus that carries features of a nuclear targeting signal (15, 16). p85\(^{S6k}\) is localized in the nucleus (17, 18), where it might phosphorylate a nuclear pool of S6 protein (19) or the cAMP-response element modulator (CREM), a transcription factor which was recently identified as a substrate of p70\(^{S6k}\) (20). Activation of p70\(^{S6k}\) in response to mitogens is associated with phosphorylation of three serines and one threonine located at the carboxyl terminus of the kinase (21). p70\(^{S6k}\) also contains additional phosphate groups that become dephosphorylated upon rapamycin treatment, leading to inactivation of p70\(^{S6k}\) (22). Each set of phosphorylation sites might be modified by distinct kinases; however, direct activators of p70\(^{S6k}/p85\(^{S6k}\) are so far unknown. Recent experiments based on the use of rapamycin (23, 24), specific phosphatidylinositol 3-kinase inhibitors such as wortmannin (25, 26) and platelet-derived growth factor receptor mutants (27) have suggested that phosphatidylinositol 3-kinase and the structurally related enzyme RAFT/FRAP are involved in upstream signaling to p70\(^{S6k}/p85\(^{S6k}\).

Two lines of evidence have suggested that the function of p70\(^{S6k}/p85\(^{S6k}\) during G\(_0\) is important for cell cycle progression. First, inhibition of p70\(^{S6k}/p85\(^{S6k}\) by treatment of cells with rapamycin leads to cell cycle arrest in G\(_0\) or a delay of entry into S phase, depending on the cell type (9, 14). Second, microinjection of rat embryo fibroblasts with antibodies that inhibit p70\(^{S6k}/p85\(^{S6k}\) abolishes the serum-induced entry into S phase (17, 28). Activation of p42\(^{mapk}/p44\(^{mapk}\) is also thought to be essential for triggering the proliferative response in fibroblasts (29). To gain further insight into how p70\(^{S6k}/p85\(^{S6k}\) and p42\(^{mapk}/p44\(^{mapk}\) activity is regulated and what role the enzymes might have in cell growth and cell cycle control, we have examined the behavior of these enzymes during the cell cycle. We show here that p70\(^{S6k}\) and p42\(^{mapk}/p44\(^{mapk}\) activities are
regulated in a cell cycle-dependent manner. Furthermore, we present evidence that the cell cycle regulation of p70S6K activity might involve compartmentation and a regulatory mechanism that is independent of the four carboxyl-terminal phosphorylation sites. Finally, our observations of the behavior of p70S6K and p42mapk/p44mapk during the cell cycle suggest that there is cross-talk between these signaling molecules and the cell cycle machinery.

MATERIALS AND METHODS

Synchronization of Cells—Swiss mouse 3T3 fibroblasts were seeded in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) containing 10% fetal calf serum (FCS, Life Technologies, Inc.) at 1.3 x 10^6 cells per 10-cm plate or 3.0 x 10^6 cells per 15-cm plate and allowed to grow for 3 days. To synchronize cells, cells were serum-starved in DMEM plus 0.5% FCS for 24-48 h. Then 5 mM epidermal growth factor (EGF), 1 mM insulin, and 6% FCS were added to induce re-entry into the cell cycle. Alternatively, 20% FCS was used as a mitogen.

To arrest cells in metaphase, cells were first presynchronized in G0 by serum starvation and then stimulated with EGF, insulin, and FCS as described above. Nocodazole (0.4 μg/ml, Sigma) was added 20 h after release from G0, before cells entered M phase. Mitotic cells were collected by centrifugation. The cells were washed twice with PBS and fixed as described above. Incubation with primary antibodies (affinity-purified anti-p70S6K antibody; dilution 1:50 in PBS or anti-tubulin monoclonal YOL1/34 antibody; dilution 1:50 in PBS) was performed overnight at room temperature. The secondary fluorescein-conjugated goat anti-rabbit IgG (G+L) (Tago, Inc.) was diluted 1:50 in PBS and incubated 1 h at room temperature in the dark. The secondary rhodamine-conjugated goat anti-rat IgG (H+L) (Jackson ImmunobioResearch) was diluted 1:1500 in PBS. Nuclear staining was performed with 0.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI). Fluorescence was visualized with a Zeiss Axioskop microscope equipped with a CCD camera. Images were processed with GeneJoin and Photoshop programs.

Western Blotting—Proteins in cell extract supernatants (5 μg samples) were separated on 15% polyacrylamide gels and electrophoretically transferred onto nitrocellulose. The membranes were blocked with 5% dried milk in PBS, 0.5% Tween 20 and then incubated with either 1:1000 diluted afffinity-purified antibody against p70S6K or 1:2000 diluted antibody against p42mapk/p44mapk (Upstate Biotechnology, Inc.). After several washes in PBS, 0.5% Tween 20, membranes were incubated with 1:5000 diluted horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham) for 1 h. Detection of the immune signal was done using the ECL kit (Amersham).

Fibroblasts Stably Expressing p70S6K Constructs—Mutant p70S6K was generated by replacing Ser→Asp residues (411, 418, and 424) and Thr→Glu (residue 421) by the polymerase chain reaction.2 Wild-type or mutant p70S6K cDNAs (nucleotides 137-1705; Ref. 4) carrying the HA tag S to the p70S6K sequence were inserted into the mammaliam expression vector pMV-7 (36). The wild-type and mutant inserts were verified by nucleotide sequencing. pmV7+ wild-type S6 kinase, pmV7+ mutant S6 kinase, or vector alone were transfected into the packaging and virus-producing cell line GP+E8 37 using calcium phosphate precipitation. Neo+ clones were selected by growth in medium containing 1 mg/ml G418 (Geneticin, Life Technologies, Inc.); clones were visible 8–10 days after selection. Preparation of virus stocks and viral infection procedures were carried out essentially as described earlier (38). Briefly, Swiss mouse 3T3 fibroblasts were infected with cell-free virus from the virus-producing cells in the presence of 8 μg/ml Polybrene. The medium was changed the following day and two days after infection cells were trypsinized and replated at a 1:3 dilution in G418 medium. Stable transfecteds were obtained 1–2 weeks after replating.

RESULTS

S6 Kinase and MAP Kinase Activity in Cells Released from G0—Most studies examining the regulation of p70S6K activity have focused on the first few hours after stimulation of G0-arrested cells. We examined the behavior of p70S6K over the course of one complete cell cycle to determine whether the enzyme is active at other times besides G1 and to compare its activity in G2/M and G/M cells. Subconfluent Swiss mouse 3T3 fibroblasts were synchronized in G0 by serum starvation and then induced to enter the cell cycle by adding FCS plus insulin and EGF. Under these conditions the cells traversed the first cell cycle with good synchrony (Fig. 1A). Analysis of cellular DNA content by flow cytometry showed that cells started to enter S phase synchronously after 16 h of stimulation and 4–6 h later 95% of the cells had doubled their DNA content. After 26–28 h of stimulation most of the cells had gone through M phase and reappeared as G1 cells with a 2 N content of DNA (Fig. 1A). Similar results were obtained using 20% FCS as a mitogen (data not shown).

To measure p70S6K activity in extracts of synchronized cells,
immunocomplex kinase assays were performed using 40 S ribosomal subunits as a substrate. In these experiments an antibody that recognizes both p70\SPS{S6K} and p85\SPS{S6K} was used. However, since p85\SPS{S6K} is much less abundant than p70\SPS{S6K} in these cells (17), most of the activity measured in the immunocomplexes is contributed by p70\SPS{S6K}. p70\SPS{S6K} activity measured 20 min after mitogen stimulation was more than 20 times higher than the activity measured in serum-starved cells (Fig. 1B, closed circles). The kinase lost 40% of its activity by the end of G1 (Fig. 1B, 16 h), consistent with published data (28). During S and G2 the activity continued to decrease, so that during M phase a relatively low level of activity was measured (Fig. 1B). It was shown earlier that p70\SPS{S6K} is activated at the first G2/M boundary during meiotic maturation of Xenopus laevis oocytes (39). However, we saw no increase in p70\SPS{S6K} activity in fibroblasts cycling from G2 into M (Fig. 1B). A slight nuclear transcription factor CREM\SPS{SPS{SPS{}} was shown to be phosphorylated by p70\SPS{S6K} (20), we also performed immunocomplex kinase assays with recombinant CREM\SPS{SPS{SPS{}} as a substrate. The pattern of p70\SPS{S6K} activity toward CREM\SPS{SPS{SPS{}} during the cell cycle was virtually identical to that seen toward S6k (data not shown).

All evidence obtained so far indicates that p70\SPS{S6K} is activated by phosphorylation (21, 40). To determine if the gradual decrease in p70\SPS{S6K} activity observed in Fig. 1B could be due to dephosphorylation, the phosphorylation state of p70\SPS{S6K} was assessed by its appearance as multiple bands on Western blots. The active, phosphorylated form of p70\SPS{S6K} migrates more slowly in SDS-polyacrylamide gels than the dephosphorylated, inactive species (40). After 20 min of stimulation the most highly phosphorylated form of p70\SPS{S6K} was detected (Fig. 1C, upper panel), which correlated to the highest S6 kinase activity (Fig. 1B, closed circles). The hyperphosphorylated form was seen throughout G2, and by the end of G2 partially dephosphorylated forms of the kinase appeared (Fig. 1C, upper panel). As p70\SPS{S6K} activity continued to decrease, the hypophosphorylated forms of the kinase became predominant. The Western analysis also showed that the amount of p70\SPS{S6K} protein remained constant during the cell cycle (Fig. 1C). Together, these data demonstrate that p70\SPS{S6K} activity is cell cycle regulated and that the changes in activity appear to be mediated by phosphorylation-dephosphorylation.

In parallel to p70\SPS{S6K}, we also examined the cell cycle regulation of MAP kinases. Immunocomplex kinase assays showed that p42\SPS{MAPK} was strongly but only transiently activated early in G1/G2 (Fig. 1B, open squares). During S and G2, p42\SPS{MAPK} activity was at basal levels and during G2/M the kinase activity increased to a small extent. MAP kinases were also visualized on Western blots that were probed with an antibody that recognizes both p42\SPS{MAPK} and p44\SPS{MAPK} (Fig. 1C, lower panel). The mitogen-dependent activation of p42\SPS{MAPK}/p44\SPS{MAPK} was clearly seen as a shift of the proteins to the phosphorylated, more slowly migrating forms after 20 min of stimulation (Fig. 1C, lower panel). Phosphorylated species of p42\SPS{MAPK}/p44\SPS{MAPK} were also seen at 4 h but thereafter the dephosphorylated forms were predominant. Because p42\SPS{MAPK} gives a relatively faint signal on Western blots, no up-shifted band that would represent active p42\SPS{MAPK} was seen in G2/M extracts (Fig. 1C, lower panel). However, a minor upper band representing active p44\SPS{MAPK} was visible at later stages in the cell cycle (Fig. 1C, lower panel).

S6 Kinase and MAP Kinase Activity in Cells Released from Metaphase—Because fibroblasts released from G2 were not well synchronized after completing the first cell cycle (Fig. 1A), a different synchronization method had to be used in order to study p70\SPS{S6K} in cells exiting mitosis and entering G1 of the next cell cycle. We explored several methods to obtain enriched populations of cells in G1/S or G2/M and the best results were obtained using a nocodazole-induced metaphase block (see “Materials and Methods”). The mitotic cells were collected with polyclonal antibodies (see “Materials and Methods”).
The cells progressed from M into G1. The activity increased more than 10-fold (Fig. 2B). Western analysis showed that the increase in activity was associated with an increase in phosphorylation of p70S6k and that the amount of expressed protein did not change (Fig. 2C, upper panel).

p42mapk also became activated in G1 cells after release from nocodazole but the activity returned very rapidly to near-basal levels (Fig. 2B, open squares). Western analysis showed that p42mapk and p44mapk were phosphorylated 1.5 h after release from nocodazole and extensively dephosphorylated 3 h after release (Fig. 2C, lower panel). These results show that p70S6k and p42mapk/p44mapk are not only activated at the G0/G1 boundary, but also in fibroblasts cycling from M into G1 after release from a nocodazole block.

One could argue that the low p70S6k activity measured in metaphase-arrested cells might be an artifact of nocodazole treatment. To exclude this possibility, we compared the amount of p70S6k activity in mitotic cells that were collected by shake-off with or without nocodazole treatment and found that there was no significant difference (Fig. 3A). In addition, there was no difference in kinase activity in the drug-treated or non-treated cells that were left on the plates after the shake-off (non-mitotic cells). B, confluent cells were treated with (+) or without (−) different concentrations of nocodazole for 30 min. Then the cells were treated with or without 5 nM EGF for 20 min. Total S6 kinase activity was measured.

Additional control experiments were performed to determine whether kinase activation might be due to withdrawal of nocodazole rather than to a specific cell cycle change. Removal of

**Fig. 2.** p70S6k and p42mapk/p44mapk in cells progressing from M phase into G1. Mitotic cells were collected after nocodazole treatment as described under “Materials and Methods” and reseeded into drug-free medium at t = 0 h. A, entry into G1 was followed by analyzing cellular DNA content by flow cytometry. B, at the indicated times, cell extract supernatants were made and p70S6k (■) and p42mapk (□) activity was measured in immunocomplex kinase assays. C, the amount of expressed protein and the phosphorylation state of p70S6k (upper panel) and MAP kinases (lower panel) were determined by Western analysis.

**Fig. 3.** Effect of nocodazole on S6 kinase activity. A, mitotic cells were collected by shake-off after treatment with (+) or without (−) nocodazole (see "Materials and Methods"). Total S6 kinase activity was measured in extract supernatants prepared from mitotic cells and from cells that were left on the plates after the shake-off (non-mitotic cells). B, confluent cells were treated with (+) or without (−) different concentrations of nocodazole for 30 min. Then the cells were treated with or without 5 nM EGF for 20 min. Total S6 kinase activity was measured.
Localization of p70<sup>SEK</sup>/p85<sup>SEK</sup> during the Cell Cycle—p70<sup>SEK</sup> and p85<sup>SEK</sup> have been reported to be differentially distributed between the nucleus and cytoplasm in cycling cells (17, 18). To determine if the localization of p70<sup>SEK</sup>/p85<sup>SEK</sup> changes during the cell cycle, the enzymes were examined by indirect immunofluorescence. In resting cells a faint staining of both cytoplasm and nucleus was seen (Fig. 5A, green). This distribution remained unchanged in cells stimulated for 45 min with 20% serum and in cells late in G<sub>1</sub>. During S phase the cytoplasm was still faintly stained but dots of p70<sup>SEK</sup>/p85<sup>SEK</sup> appeared in the nucleus in a pattern similar to that seen with DAPI DNA staining (Fig. 5A, blue). The speckled appearance of p70<sup>SEK</sup>/p85<sup>SEK</sup> staining in the nucleus was even more striking in G<sub>2</sub> cells (Fig. 5A, green). As the nuclear signal became more intense and less exposure time was required to produce the photographs, staining of the cytoplasm seemed to fade; however, it was still almost the same in G<sub>2</sub> cells as in resting cells. During mitosis most of the p70<sup>SEK</sup>/p85<sup>SEK</sup> signal overlapped with DAPI staining but the cytoplasm was still faintly stained (Fig. 5A).

We also examined the distribution of p70<sup>SEK</sup>/p85<sup>SEK</sup> in cells released from a nocodazole block. As was seen in Fig. 5A, the kinase colocalized with DNA during mitosis (Fig. 5B). In very early G<sub>1</sub>, after cytokinesis had occurred and the DNA had decondensed, the speckled S6 kinase pattern appeared in the nucleus (Fig. 5B, t = 2 h) and then disappeared 1 h later. Thus, p70<sup>SEK</sup>/p85<sup>SEK</sup> is localized in the cytoplasm at all times during the cell cycle but becomes enriched at certain locations in the nucleus during S/G<sub>2</sub> phase and early G<sub>1</sub> cells released from a metaphase block.

Constitutive Signaling to p70<sup>SEK</sup> during the Cell Cycle—p70<sup>SEK</sup> remains active to various extents during the entire cell cycle (Fig. 1B). One explanation for this might be that the activated kinase is stable and remains active for hours. Alternatively, the activity of the kinase might depend on constitutive signaling from extracellular growth factors. To discriminate between these two possibilities, mitogenes were removed from cells in different stages of the cell cycle and the effect on p70<sup>SEK</sup> activity was examined. Serum-starved cells were induced to enter the cell cycle synchronously as described before. As a control, one set of cells was left without any further treatment. A second set of cells was incubated at various times without FCS for 10 min, while a third set was incubated first without FCS and then with FCS for 20 min. Removal of serum after 20 min of mitogen stimulation did not lead to a decrease in p70<sup>SEK</sup> activity (Fig. 6A, compare light gray with dark gray bars). However, withdrawal of growth factors at later times or from asynchronously cycling cells led to a significant reduction in p70<sup>SEK</sup> activity (Fig. 6A). Western analysis showed that the decrease in p70<sup>SEK</sup> activity correlated with a loss of the most highly phosphorylated form of the kinase (Fig. 6B). The kinase could be reactivated at any time by readdition of FCS; however, it was only activated to the level that is characteristic for a particular cell cycle stage, and never to the high level seen in early G<sub>1</sub> (Fig. 6A, compare dark gray and open bars). These data show that signaling to p70<sup>SEK</sup> is constitutively on throughout the cell cycle. The reactivation data suggest that a negative regulatory mechanism is present during later parts of the cell cycle or that a component of the S6 kinase signaling pathway becomes limiting.

Involvement of Carboxyl-terminal Phosphorylation Sites in Regulating p70<sup>SEK</sup> Activity during the Cell Cycle—p70<sup>SEK</sup> was activated to a lower extent at the M/G<sub>1</sub> transition as compared to G<sub>0</sub>/G<sub>1</sub> (Figs. 1B and 2B), suggesting the possibility that different phosphorylation sites might be involved in activating
the kinase during these two stages of cell cycle. As the carboxyl-terminal phosphorylation sites display a Ser/Thr-Pro motif that is recognized by cell cycle-regulated kinases (21), we examined the contribution of these phosphorylation sites to the cell cycle regulation of p70S6k activity. A mutant p70S6k was constructed in which the three serines were mutated to aspartic acid and the threonine to glutamic acid (Fig. 7A). These changes were introduced to mimic phosphorylation. To distinguish between endogenous and exogenous kinase, an HA epitope tag was added to the amino terminus of the protein (Fig. 7A). Constructs encoding the tagged wild-type and mutant p70S6k were used to produce fibroblasts stably expressing these proteins. Sequential immunoprecipitation of cell extracts with antibodies to the HA tag and then to p70S6k, followed by S6 kinase assays of the immunoprecipitates, suggested that the recombinant proteins were present in low amounts as compared to the endogenous p70S6k (see legend to Fig. 7B). In addition, the exogenous kinases were not detectable on Western blots probed with HA antibodies (data not shown).

If the four carboxyl-terminal phosphorylation sites are responsible for regulating the activity of p70S6k during the cell cycle, the mutant kinase should display the same level of activity at all times. To test this prediction, mitotic and G1 populations of fibroblasts were collected and the tagged wild-type and mutant p70S6k were assayed in HA immunoprecipitates. The activity of recombinant wild-type p70S6k was low in mitotic cells and increased when cells entered G1 (Fig. 7B). However, the mutant enzyme also became more active as cells moved from M phase into G1. No p70S6k activity was immunoprecipitated from cells transfected with the empty vector (Fig. 7B). To further characterize the behavior of the mutant p70S6k, we examined its ability to respond to mitogens in G0 cells. Confluent fibroblasts were treated with or without EGF and the tagged kinases were assayed in HA immunoprecipitates. Both the wild-type and mutant kinases were activated upon addition of EGF (Fig. 7B). The degree of EGF-induced activation of the HA-tagged kinases was significantly lower than that seen with endogenous p70S6k in the parental cells (Fig. 3B). We therefore measured the activity of endogenous p70S6k in the transfected cells by adding p70S6k antibodies to supernatants that had been preincubated with HA antibody. Assay of these immunoprecipitates showed that activation of the endogenous p70S6k in pMV7-transfected cells was also reduced (2.2–5.1 fold; see legend to Fig. 7B).

Having established that the mutant p70S6k could still be activated during M/G1 and G0/G1, we asked if the mutant enzyme was also sensitive to negative regulators of the p70S6k pathway such as wortmannin (27) and rapamycin (14). Indeed, treatment of transfected fibroblasts with wortmannin or rapamycin completely abolished the EGF-induced activation of both mutant and wild-type p70S6k (Fig. 7B). In addition, similar to the results obtained with endogenous p70S6k in the parental cells (Fig. 6), withdrawal of FCS from transfected cells in S phase led to a rapid decline in S6 kinase activity of the mutant protein (Fig. 7B). Thus, the apparently normal regulation of the p70S6k mutant suggests that the carboxyl-terminal phospho-
mitosis appears to suppress the negative regulatory mechanism, thus creating an environment that is permissive for kinase activation. This negative regulation of p70S6K and p42\textit{mapk}/p44\textit{mapk} might be mediated by a component of the cell cycle machinery such as a cyclin, which accumulates until the end of M phase and is then rapidly degraded.

p70\textit{S6K} and p42\textit{mapk}/p44\textit{mapk} were activated more strongly during the G0/G1 transition (Fig. 1B) than during M/G1 (Fig. 2B). This difference is probably linked to the differential protein synthesis requirement of these cells. Quiescent cells contain fewer ribosomes and synthesize proteins at a lower rate than cycling cells (41). A sustained increase in protein synthesis is required for rested cells to synthesize new ribosomes and other proteins essential for entry into S phase (42), and as a result the G1 phase is approximately 4 h longer than G0 of cycling cells. Since S6 phosphorylation enhances the synthesis of certain proteins involved in translation (2), it seems consistent that cells moving from a quiescent state into S phase would require higher levels of S6 kinase activity than M/G1 cells. In addition, an essential function of MAP kinases in G1 might be to phosphorylate an inhibitory subunit of translation initiation factor eIF-4E, thereby stimulating the overall rate of protein synthesis in response to mitogens (43).

Similar to our results, Tamemoto et al. (44) found that p42\textit{mapk}/p44\textit{mapk} activity is low in nocodazole-arrested Chinese hamster ovary cells and that the enzymes become active upon entry into G1. Furthermore, p42\textit{mapk}/p44\textit{mapk} were re-activated at around M phase of the next cell cycle (44). The interpretation of these results was that p42\textit{mapk}/p44\textit{mapk} are activated biologically, first in G1 and then in G1/M before the nocodazole arrest point. However, loss of synchrony by the end of the first cell cycle would make it impossible to determine whether activation occurred in G1/M or G1. It has been proposed that activation of MAP kinases in G1 might be a one-time event involved in releasing cells from an arrested state, rather than a recurring event required for progression through each cell cycle (45). This hypothesis is based mainly on experiments done with oocytes and early embryos. However, MAP kinase activation at M/G1 in fibroblasts (Figs. 2 and 4) and Chinese hamster ovary cells (44) suggests that the requirement for MAP kinase activity during G1 might be different in rapidly dividing embryonic cells and in established cell lines.

Nuclear Localization of p70S6K/p85S6K—Compartmentation is known to be an important mechanism that can regulate protein function. The presence of a nuclear localization signal in p85S6K but not p70S6K has prompted a number of studies examining the intracellular distribution of these enzymes (17, 18, 20). A summary of the results obtained is that while p70S6K is mainly cytoplasmic and p85S6K is mainly nuclear, their presence in the alternative compartment cannot be ruled out. Our examination of the distribution of p70S6K/p85S6K during the cell cycle revealed that bright speckles of p70S6K/p85S6K staining appeared in the nucleus of S phase cells, yielding an appearance similar to that provided by DAPI DNA staining (Fig. 5A). Staining of the cytoplasm remained about the same, so the increased signal in the nucleus might be due to concentration of p70S6K/p85S6K into localized spots, as opposed to an influx of cytoplasmic enzyme. Since the antibody used in these experiments detects both p70S6K and p85S6K, no statement can be made about which isoform is responsible for the increased nuclear signal. However, we have detected a major band corresponding to p70S6K on Western blots of cytoplasmic and nuclear fractions prepared from cells in different phases of the cell cycle (data not shown). During mitosis p70S6K/p85S6K colocalized with chromosomes (Fig. 5). Components of small nuclear ribonucleoprotein particles show a speckled staining pattern of...
similar to the one seen here, but the speckles do not overlap with DAPI staining and the small nuclear ribonucleoprotein proteins do not migrate with DNA during mitosis (46). Therefore, p70\textsuperscript{65k} is not colocalized with RNA splicing centers but rather with heterochromatin, which is stained by DAPI. Interestingly, the speckly distribution of p70\textsuperscript{65k} in the nucleus did not seem to correlate with S6 kinase activity or with the overall phosphorylation state of p70\textsuperscript{65k}, since the nuclear speckles were seen in both G\textsubscript{2} and early G\textsubscript{1} cells (Figs. 1, 2, and 5).

Ribosomal protein S6 is present in both the cytoplasm and nucleus, where it is found in nucleoli and in association with chromatin (19). The nucleolar pool of S6 has been shown to become phosphorylated in response to treatment of cells with phorbol esters (19). Since protein synthesis does not take place in the nucleus, the function of phosphorylated S6 protein in this compartment remains obscure. Although it has been shown that p85\textsuperscript{65k} activity in the nucleus is required for entry into S phase (17), it is not known whether the essential function of this enzyme is to phosphorylate S6 or another substrate.

Constitutive Signaling to p70\textsuperscript{65k}-p70\textsuperscript{65k} Activity Depends on Permanent Signaling from Extracellular Growth Factors. Removal of mitogens at any time during the cell cycle leads to a rapid inactivation of the enzyme that can be reversed by adding back growth factors (Fig. 6). Thus, the components of at least one signaling pathway leading to p70\textsuperscript{65k} are always present and capable of transducing signals. However, the kinase cannot be reactivated to the high level measured in G\textsubscript{1} cells, indicating that signaling is attenuated during later stages of the cell cycle. Similar results were obtained with p42\textsuperscript{mapk} (data not shown). Cell cycle-dependent attenuation of the kinase activation pathways could be accomplished by several mechanisms.

First, the activity of growth factor receptors might be regulated during the cell cycle. It has been shown in receptor overexpression experiments that there is a strong correlation between insulin receptor kinase activity and the activities of kinases lying downstream in the signaling cascade (47). Receptor activity could be down-regulated by a decrease in receptor number mediated by ligand-induced receptor internalization and degradation (48) or by a growth-dependent repression of receptor transcription (49). Alternatively, the activity of growth factor receptors might be reduced by production of inhibitors (50) or by post-translational modification (51). Another explanation for the attenuation of signaling to p70\textsuperscript{65k} might be that a component in the signaling cascade downstream of the receptor becomes rate-limiting. Finally, a negative regulator of p70\textsuperscript{65k} such as a phosphatase or an inhibitory subunit might become synthesized or activated as cells proceed through the cell cycle.

Involvement of Carboxyl-Terminal Phosphorylation Sites in the Cell Cycle Regulation of p70\textsuperscript{65k}. Phosphorylation-dephosphorylation is the only mechanism known at this time to regulate p70\textsuperscript{65k} activity (21, 40). It was proposed that phosphorylation of four amino acids at the carboxyl terminus of p70\textsuperscript{65k} might be responsible for mitogen-induced enzyme activation at G\textsubscript{2}/G\textsubscript{1} (21). However, a recent publication (52) that appeared after completion of this work showed that a p85\textsuperscript{65k} mutant with 104 amino acids deleted from the carboxyl terminus could still be activated by FCS in G\textsubscript{0} cells and inhibited by rapamycin and wortmannin. Furthermore, it was proposed that kinase activation might result from the phosphorylation of Thr-252 in response to a signal generated by phosphatidylinositol 3-kinase (53). Our results using a p70\textsuperscript{65k} molecule with more subtle point mutations confirm that p70\textsuperscript{65k} can be activated independently of the carboxyl-terminal phosphorylation
sites in G_{1}/G_{0}. In addition, our examination of the behavior of this p70^{eq}_{5k} mutant at M/G_{1} and during S phase suggests that these phosphorylation sites are also not involved in controlling kinase activity during other phases of the cell cycle (Fig. 7B). The specific mechanisms that contribute to the cell cycle-dependent regulation of p70^{eq}_{5k} activity remain to be determined.

We have shown that the behavior of p70^{eq}_{5k}/p85^{eq}_{5k} and p4^{eq}_{220}p4^{eq}_{220} is cell cycle-regulated. Because the activities of these enzymes are sensitive to changes in the growth factor supply, one might imagine that the kinases are part of a sensory system that evaluates growth conditions and makes the decision to exit or remain in the cell cycle. A tightly controlled interplay between these signal transduction molecules and the cell cycle machinery might be important for ensuring proper cell growth and proliferation.

Acknowledgments—We thank G. Thomas for the p70^{eq}_{5k} cDNA, M. Buslliinger for pMV-7, J. Kilmartin for YOL134 antibody, C. Marshall for p4^{eq}_{220}p4^{eq}_{220} antibody, and P. Sassone-Corsi for recombinant CREM protein. We are grateful to R. Kurzbauer for sequencing, H. Tkadletz for help with the figures, and C. Koch and H. Beug for comments on the manuscript.