De novo pyrimidine biosynthesis is activated in proliferating cells in response to an increased demand for nucleotides needed for DNA synthesis. The pyrimidine biosynthetic pathway in baby hamster kidney cells, synchronized by serum deprivation, was found to be up-regulated 1.9-fold during S phase and subsequently down-regulated as the cells progressed through the cycle. The nucleotide pools were depleted by serum starvation and were not replenished during the first round of cell division, suggesting that the rate of utilization of the newly synthesized nucleotides closely matched their rate of formation. The activation and subsequent down-regulation of the pathway can be attributed to altered allosteric regulation of the carbamoyl-phosphate synthetase activity of CAD (carbamoyl-phosphate synthetase-aspartate carbamoyltransferase-dihydroorotase), a multifunctional protein that initiates mammalian pyrimidine biosynthesis. As the cycle approached S-phase there was an increased sensitivity to the allosteric activator, 5-phosphoribosyl-1-pyrophosphate, and a loss of UTP inhibition, changes that were reversed when cells emerged from S phase. The allosteric regulation of CAD is known to be modulated by MAP kinase (MAPK) and protein kinase A (PKA)-mediated phosphorylations as well as by autophosphorylation. CAD was found to be fully autophosphorylated in the synchronized cells, but the level remained invariant throughout the cycle. Although the MAPK activity increased early in G1, the phosphorylation of the CAD MAPK site was delayed until just before the onset of S phase, probably due to antagonistic phosphorylation by PKA that persisted until late G1. Once activated, pyrimidine biosynthesis remained elevated until rephosphorylation of CAD by PKA and dephosphorylation of the CAD MAPK site late in S phase. Thus, the cell cycle-dependent regulation of pyrimidine biosynthesis results from the sequential phosphorylation and dephosphorylation of CAD under the control of two important signaling cascades.

The activity of the de novo pyrimidine biosynthetic pathway closely parallels the growth rate of the cell and is highest during periods of rapid proliferation (1–13). Cells growing in culture synthesize pyrimidines at a rapid rate during the exponential growth phase, but the pathway is much less active when the cells enter the stationary phase or have their growth arrested by serum deprivation (14–18). In an earlier study, Mitchell and Hoogenraad (19) show that pyrimidine biosynthesis is maximally activated during the S phase of the cell cycle. Thus, the de novo biosynthetic pathway plays a dominant role in providing precursors for the synthesis of DNA that accompanies cell division. Furthermore, there is strong evidence (7, 11, 12) that the activation of the pathway is a prerequisite for the proliferation of tumor and neoplastic cells.

The flux through the pathway is governed (20, 21) by the activity of carbamoyl-phosphate synthetase II (CPSase), 1 a component of CAD (Fig. 1), the multifunctional protein (22–24) that initiates pyrimidine biosynthesis in mammalian cells. CPSase catalyzes the rate-limiting step, the formation of carbamoyl phosphate from bicarbonate, glutamine, and two ATP molecules. The enzyme is subject to a complex network of control mechanisms that include both allosteric regulation and phosphorylation. UTP, an end product of the pathway, is a feedback inhibitor, whereas 5-phosphoribosyl-1-pyrophosphate (PRPP), a substrate for both the parallel de novo purine biosynthetic pathway and a subsequent step in pyrimidine biosynthesis, is an activator (13, 25, 26). Carrey et al. (28-31) made the important observation that purified CAD is phosphorylated at two sites (Fig. 1) by cAMP-dependent protein kinase A (PKA). PKA phosphorylation has no effect on the catalytic activity but decreases UTP inhibition, a modification that would be expected to stimulate pyrimidine biosynthesis. However, subsequent studies (32) showed that PKA phosphorylation resulted in a decrease in the affinity for the activator, PRPP, an effect that would be expected to down-regulate the pathway. Thus, PKA phosphorylation alone cannot account for the activation of the pathway in rapidly growing cells.

MAP kinases (33) are ubiquitous components of the mitogen-activated cascade that result in cellular proliferation in response to growth factors. Stimulation of quiescent cells with epidermal growth factor results in the activation of the MAP kinase cascade and the phosphorylation of Thr-456 in the A1 subdomain (Fig. 1) of the CAD CPSase (34). MAP kinase-phosphorylated A1 subdomain is a substrate for both the parallel de novo pyrimidine biosynthetic pathway and a subsequent step in pyrimidine biosynthesis, is an activator (13, 25, 26). Carrey et al. (28-31) made the important observation that purified CAD is phosphorylated at two sites (Fig. 1) by cAMP-dependent protein kinase A (PKA). PKA phosphorylation has no effect on the catalytic activity but decreases UTP inhibition, a modification that would be expected to stimulate pyrimidine biosynthesis. However, subsequent studies (32) showed that PKA phosphorylation resulted in a decrease in the affinity for the activator, PRPP, an effect that would be expected to down-regulate the pathway. Thus, PKA phosphorylation alone cannot account for the activation of the pathway in rapidly growing cells.

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1 The abbreviations used are: CPSase, carbamoyl-phosphate synthetase; CPSase, the CAD domain that catalyzes carbamoyl phosphate synthesis from NH₃, bicarbonate, and ATP; CAD, the multifunctional protein that has CPSase, aspartate transcarbamoylase, and dihydroorotase activities; Erk1 and Erk2, extracellular signal-regulated kinases 1 and 2; MAPK, mitogen-activated protein (MAP) kinase; MEK, MAP kinase kinase, the kinase in the MAP kinase cascade that phosphorylates and activates MAP kinase; PKA, cAMP-dependent protein kinase A; HPLC, high-performance liquid chromatography; DMEM, Dulbecco’s modified Eagle’s medium; BHK cells, baby hamster kidney cells; PRPP, 5-phosphoribosyl-1-pyrophosphate; ATC, the CAD domain that catalyzes the formation of dihydroorotate; GLN, the CAD domain that hydrolyzes glutamine and transfers the ammonia to the CPS domain.
phorylation both in vivo and in vitro has no effect on any of the catalytic activities of CAD but relieves UTP inhibition and stimulates PRPP activation of the CPSase. Both of these changes in allosteric regulation would be expected to increase the rate of pyrimidine biosynthesis.

We have recently shown (18) that the activation of pyrimidine biosynthesis in exponentially growing BHK 165-23 cells is a consequence of the MAP kinase-mediated phosphorylation of CAD and that its subsequent down-regulation as the culture became confluent is associated with dephosphorylation of the MAP kinase site and phosphorylation by PKA. To more precisely define the mechanism of growth-related activation and down-regulation of pyrimidine biosynthesis, the activity of the pathway and the role of the diverse control mechanisms were examined as BHK 165-23 cells traversed the cell cycle.

EXPERIMENTAL PROCEDURES

Cell Culture—BHK 165-23 (35), a baby hamster kidney cell line derived from BHK-21, was grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% dialyzed fetal bovine serum and 2 μg/ml gentamicin (Invitrogen). Two million cells were plated in T75 flasks containing 25 ml of medium. The medium was changed every 2 days. Cells were counted using a hemocytometer, and viability was assessed by trypan blue staining.

Cell Synchronization—BHK cells were synchronized by serum deprivation as described previously (36). Briefly, 10 T-75 flasks of exponentially growing cells (60–80% confluent) were serum-starved in DMEM, 0.1% fetal bovine serum for 24 h. The cells were then stimulated by the addition of 25 ml of DMEM supplemented with 10% fetal bovine serum and incubated at 37 °C. The cultures were harvested at 2-h intervals over a period of 20–24 h for pyrimidine biosynthesis assays and the preparation of cell extracts as previously described (18).

Pyrimidine Biosynthesis—The rate of pyrimidine biosynthesis in live cells was assayed using a modification of the methods devised by Huisman et al. (1) and Simmonds and co-workers (37). Cells were harvested by trypsin treatment and suspended at a concentration of 2.5 × 10^6 cells/ml in 2.8 ml of DMEM, F-12 medium without bicarbonate. The assay was initiated by the addition of 0.33 ml of 49 mM 14C-labeled sodium bicarbonate (1.6 Ci/mmol) equilibrated with 5 mM ammonium phosphate buffer at pH 7.2 (Buffer A). The nucleotides were eluted with a discontinuous gradient of 5 mM (NH4)2PO4, pH 7.2 (Buffer A), and 750 mM (NH4)2PO4, pH 3.8 (Buffer B), at a flow rate of 2 ml/min. Buffer B increased linearly to 50% in 20 min and then from 50 to 100% over the next 10 min. The elution was then continued at 254 and 280 nm using the radioactivity flow detector. The peaks were integrated using the UN-4 systems software.

The identity and total amount of each nucleotide were calculated from standard curves obtained using authentic standards. The amount of the radiolabeled nucleotides were calculated from the specific radioactivity of the 14C-labeled sodium bicarbonate.

Immunoblotting—For immunoblotting the following antibodies were used at the previously described dilution (18) or as recommended by the manufacturer: phosphoserine (Z PS-1, Zymed Laboratories Inc.), rabbit polyclonal antibodies and phosphothreonine (Z PT-1, Zymed Laboratories Inc.) rabbit polyclonal antibodies; diphospho-Erk1/2 (anti-Thr-202/ Tyr-204-phosphorylated p44/p42, Cell Signaling Technology) mouse monoclonal antibody; PKA (C-20, sc-1304, Santa Cruz) rabbit polyclonal antibody; affinity-purified goat anti-mouse IgG (H+L) antibody conjugated to horseradish peroxidase and affinity-purified goat anti-rabbit IgG (H+L) antibody conjugated to horseradish peroxidase (Cell Signaling Technology); rabbit polyclonal antibody against all Aequorea victoria green fluorescent protein variants (Living Colors A.v., Clontech); cyclin E (Upstate Biotechnology) rabbit polyclonal IgG; cyclin B1 (clone CB169, Upstate Biotechnology) mouse monoclonal IgG; cyclin A (Upstate Biotechnology) rabbit polyclonal IgG; purified mouse anti-human RB protein (Pharmingen). The rabbit polyclonal CAD serum was prepared as previously described (38). Immunoblotting and quantitation of the immunoblots were carried out as previously described (18).

Extracellular Degradation Assay—The glucose-deprived serum-free conditioned media described (39). The 1-ml assay mixture contained 100 μg of protein, 100 mM Tris-HCl, pH 8.0, 100 μM KCl, 7.5% MeSO, 2.5% glycerol, 1 mM dithiothreitol, 3.5 mM glutamine, 20.2 mM aspartate, 1.5 mM ATP, 3.5 mM MgCl2, and 5 mM sodium 14C-labeled bicarbonate (1.6 × 10^9 cpm/mmol). The concentration of MgCl2 was adjusted so as to maintain a 2 mM excess over the sum of the concentration of ATP, UTP, and PRPP. Aspartate transcarbamoylase activity was assayed by the colorimetric method (40). The aspartate transcarbamoylase assay mixture contained 100 μg of protein, 5 mM carbamoyl phosphate, and 12 mM aspartate in a buffer consisting of 100 mM Tris-HCl, pH 8.0, 100 mM KCl, 7.5% MeSO, 2.5% glycerol, and 1 mM dithiothreitol in a total volume of 1 ml. The MAP kinase activities were determined using New England Biolabs kit following the manufacturer's protocol. The phosphorylation of the MAP kinase substrate, glutathione S-transferase-Elk1 (2 μg), was followed by Western blotting using specific anti-phospho-Elk1 antibodies (18). Alternatively, Erk1/2 were assessed using phospho-Erk1/2 antibodies that recognize only the activated kinases (Promega). The PKA activity in the cell extract (20–150 μg of protein) was assayed using the PepTag PKA assay kit (Promega), which relies on a change in the electrophoretic mobility of a fluorescent Leu-Arg-Arg-Ala-Ser-Leu-Gly peptide (Kemptide) derivative upon phosphorylation. Phosphorylation of the Kemptide could also be measured by electrophoresis on a 20% SDS-PAGE followed by immunoblotting using anti-phospho-serine antibodies.

Clone Phosphorylation Assay—Phosphorylation activity was assayed to determine the extent of the phosphorylation of the MAP kinase site, Thr-456, which could be quantitated by immunoblotting with phosphothreonine antibodies corrected for autophosphorylation. Similarly, the extent of phosphorylation of the CAD PKA sites (Ser-1406 and Ser-1859) could be quantitated using the phosphoserine antibodies. Autophosphorylation was measured using previously described modification (41). The "kinase assay" was performed by incubating 0.5 ml of the kinase assay" (42). The increase in phosphorylation of CAD, determined by quantitative immunoblotting with phosphothreonine antibodies, that occurred upon incubation of the protein with 0.5 mM ATP for 2 h was taken as a measure of the unmodified autophosphorylation site of CAD in cell extracts. Purified CAD incubated with and without ATP was examined to determine the fraction of the kinase activity. Protein quantitation was performed by the Lowry method (43) and by scanning stained SDS-polyacrylamide gels calibrated with known amounts of bovine serum albumin as a standard using the software UNSCAN-IT (Silk Scientific Corp.).
Cloning of the Fluorescent Fusion Proteins—A commercial PKA clone (GeneStorm human PKA catalytic subunit from ResGen) was amplified by PCR using the primers 5′-taaagttagctggtgggacagaggggccgcaagagag-3′, incorporating a KpnI restriction site upstream of the first codon, and 5′-tttttgctgtaaatactcttgcaacctg-3′, incorporating a BclI restriction site downstream of the coding sequence. The purified PCR product was cleaved with KpnI and BclI (Invitrogen). The vector pCruz-GFP (Santa Cruz Biotechnology) was digested using KpnI and BglII and treated with thermosensitive alkaline phosphatase (Invitrogen). The purified fragments were ligated and transformed into Escherichia coli DH5α (Invitrogen). The plasmid encoded the PKA catalytic subunit with the green fluorescent protein appended to the amino end. The plasmid NptT5.5 Erk2 containing the murine Erk2 cDNA fragment, a generous gift of Dr. Melanie Cobb (Dept. of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX), was digested with NcoI and filled in with Klenow (Invitrogen). The purified fragment was digested with XhoI (New England Biolabs) and repurified. The vector pECFP-C1 (Clontech) was cleaved with BspE1, filled in with Klenow, digested with XhoI, and dephosphorylated with thermosensitive alkaline phosphatase. The vector and insert were ligated and transformed into DH5α cells. The construct encodes MAPK (Erk2) with the enhanced cyan fluorescent protein fused to the amino end. To construct the glutaminase (GLN) CPS fusion protein, pCK-CAD10 (39) encoding the full-length CAD cDNA in E. coli was cleaved with BamHI (a unique site introduced at the 3′ end of the coding sequence) and BstXI for 2 h at 37°C. The resulting fragment encoding the entire GLN-CPSase domain (Fig. 1) was purified, filled in with Klenow, and inserted into the pECFP-C1 vector (Clontech) that had been linearized by cleavage with BspE1 and filled-in. The ligation products were transformed into DH5α-subcloning efficiency bacteria, and recombinants were verified by restriction digests and sequencing. The construct pECFP-GLN-CPS, encoded CAD GLN-CPS with the enhanced cyan fluorescent protein fused to the amino end. For the construction of the full-length CAD fusion protein, pCK-CAD10 was cleaved with XhoI. The 3750-bp fragment containing the end of the CPS.B domain and the remainder of the CAD-coding sequences was inserted into pECFP-GLN-CPS that had been cleaved with XhoI. XhoI cleaves pECFP-GLN-CPS in the CPS.B domain and in the coding stream of the insert. The ligation products were transformed into DH5α. The construct, pECFP-CAD, encoded the entire CAD protein with the enhanced cyan fluorescent protein appended to the amino end. The fidelity of all constructs was verified by DNA sequencing (Wayne State University DNA Sequencing Facility).

Transfection and Fluorescence Microscopy—The E. coli transformants were cultured overnight in Luria broth, 100 μg/ml kanamycin, and plasmids were purified using the Plasmid Maxi kit (Qiagen). BHK cells were transfected with LipofectAMINE Plus reagents (Invitrogen) using 2 μg of the DNA following the manufacturer’s protocol. After 5 h, the transfection medium was replaced with DMEM containing 10% FCS and in the CPS.B domain and in the coding stream of the insert. The ligation products were transformed into DH5α. The construct, pECFP-CAD, encoded the entire CAD protein with the enhanced cyan fluorescent protein appended to the amino end. The fidelity of all constructs was verified by DNA sequencing (Wayne State University DNA Sequencing Facility).

RESULTS

Cell Synchronization—Serum deprivation following well established protocols (36) was used to synchronize cultures of BHK165-23 cells. Replacing the complete media in exponentially growing cultures of the same media supplemented with only 0.1% serum resulted in the arrest of growth. Growth resumed 24 h later upon stimulation with 10% fetal bovine serum, at which point the cells began to traverse the cell cycle in synchrony. The periodic expression of cyclins is commonly used to identify the different cell cycle phases (45–47). In this study, cell cycle-dependent markers were used to monitor the progression of the culture through the initial phases of the cycle. Cyclin E expression, known to be confined to the G1 phase, began to increase soon after serum stimulation, peaked at 4 h, and then declined. The inactivation of the retinoblastoma tumor suppressor protein by hyperphosphorylation is also known to occur during G1 (46) and to remain phosphorylated through mitosis. In the synchronized BHK cultures, retinoblastoma phosphorylation preceded the expression of cyclin E, peaking at about 5 h, remained phosphorylated for 16 h, and then decreased. Cyclin A expression, reported to begin at the G1/S boundary and to reach the highest levels in late S phase (46, 47), peaked at 12 h in BHK cells, whereas cyclin B, another S phase marker, peaked at about 13 h (Fig. 2). We concluded that the midpoint of the S phase of the cycle occurred at 10–11 h after serum stimulation.

Pyrimidine Biosynthesis—The activity of the pyrimidine biosynthetic pathway was assayed by measuring the incorporation of 14C-labeled bicarbonate into UTP and CTP. The basal level of pyrimidine biosynthesis, measured during serum deprivation (Fig. 3, 0 h) was 1.0 pmol/min/10⁶ cells. The level remained constant until just before entry into the S phase of the cell cycle, at which point the rate increased to 1.85 pmol/min/10⁶ cells. The rate of pyrimidine biosynthesis decreased as the cells emerged from S phase. Thus, there was a strong correlation between the activity and the S phase of the cell cycle. Radiolabeled bicarbonate is also a precursor of purines, and there was a parallel increase in the incorporation of 14C-labeled bicarbonate into ATP from 1.6 to 3.0 pmol/min/10⁶ cells in mid-S phase.

The Intracellular Concentration of the Nucleotides—The intracellular concentration of the nucleotides (Table I) was measured by HPLC as the cells traversed the cell cycle. The absorbance at 254 nm was monitored (Fig. 4), and the amount of each nucleotide was determined from the area of the corresponding peaks using standard curves. The concentration was calculated assuming 1 μl of intracellular fluid/10⁶ packed cells (48). Although most of the nucleotides were well separated (Fig. 4), in the best cases CTP appeared as a small leading shoulder of the
larger UTP peak. Because it proved impractical to reliably distinguish these species, the pyrimidine trinucleotide concentrations were expressed as a combined UTP and CTP.

In serum-starved cells (t = 0 h), the concentration of UTP/CTP was 0.17 mM and remained reasonably constant for the duration of the cell cycle. This value was approximately half that observed in exponentially growing cultures of the same cells. A similar trend was observed for most of the other nucleotides. Notably, the ATP concentration in the synchronized cells was 5-fold lower than the value measured in the exponentially growing cells and did not significantly increase for 18 h post-serum stimulation. The observation that the nucleotide pools did not expand during the first round of cell division despite ongoing pyrimidine biosynthesis suggests that the newly synthesized nucleotides are utilized as rapidly as they are produced.

**Fig. 3. Pyrimidine biosynthesis in synchronized BHK cultures.** The rate of pyrimidine biosynthesis in the cell was determined by measuring the incorporation of [14C]-labeled bicarbonate into UTP and CTP. Extracts of 1.8 × 10^6 cells harvested at the indicated times after serum stimulation were analyzed by HPLC as described under "Experimental Procedures." The shaded area of the bar at the top of the figure represents the S phase of the cell cycle.

**Table I**  
Intracellular nucleotide triphosphate concentrations

| Nucleotide concentration | Exponentially growing cells | Synchronized cells (hours post-serum stimulation) |
|--------------------------|-----------------------------|--------------------------------------------------|
|                          | mM                          | 0 h      | 12 h     | 18 h      |
| ATP                      | 2.87                        | 0.556    | 0.532    | 0.653    |
| GTP                      | 0.388                       | 0.062    | 0.065    | 0.051    |
| UTP + CTP                | 0.335                       | 0.166    | 0.122    | 0.146    |
| ADP                      | 1.66                        | 0.126    | 0.235    | 0.378    |
| GDP                      | 0.174                       | 0.023    | 0.040    | 0.063    |
| UDP                      | 0.103                       | 0.014    | 0.021    | 0.034    |
| UDP-sugars               | 1.046                       | 0.143    | 0.237    | 0.337    |

**Fig. 4. Determination of intracellular nucleotide concentrations by HPLC.** A 100-μl aliquot corresponding to 5.6 × 10^6 cells of an extract from an exponentially growing culture was fractionated on a Whatman Partisil 10 SAX column eluted with a discontinuous gradient of ammonium phosphate as described under "Experimental Procedures." The absorbance of the eluant was monitored at 254 nm, and the nucleotides were identified by chromatography of authentic standards. With the exception of UTP and CTP, which coeluted, all of the nucleotide tri- and diphosphates were well resolved. Several peaks in the profile remained unidentified. The nucleotide concentration was determined from the peak areas using standard curves.

**Fig. 5. CAD CPSase activity and regulation.** Panel A, the CPSase activity (○) was assayed in extracts of cells harvested at the indicated times after serum stimulation. The percent phosphorylation of the CAD autophosphorylation site (Thr-1037) was measured in the same extracts (○) by the back phosphorylation method described under "Experimental Procedures." Panel B, the effect of 0.5 mM UTP (■), 50 μM (●), and 200 μM (○) PRPP on the CPSase activity in cell extracts isolated from the synchronized cells.

In serum-starved cells (t = 0 h), the concentration of UTP/CTP was 0.17 mM and remained reasonably constant for the duration of the cell cycle. This value was approximately half that observed in exponentially growing cultures of the same cells. A similar trend was observed for most of the other nucleotides. Notably, the ATP concentration in the synchronized cells was 5-fold lower than the value measured in the exponentially growing cells and did not significantly increase for 18 h post-serum stimulation. The observation that the nucleotide pools did not expand during the first round of cell division despite ongoing pyrimidine biosynthesis suggests that the newly synthesized nucleotides are utilized as rapidly as they are produced.

**CAD Activity and Regulation**—The activity of CAD CPSase, measured in the absence of allosteric ligands (Fig. 5A), increased ~2-fold from 1.2 nmol/min immediately after serum stimulation to 2.9 nmol/min at 22 h. We have shown previously (41) that when purified CAD is incubated with ATP, the CPS domain undergoes autophosphorylation, a phenomenon that is accompanied by a 2-fold increase in the CPSase-specific enzymatic activity. The CAD autophosphorylation, measured in synchronized cultures of BHK cells (Fig. 5A), was found to be 90–95% complete and remained unchanged throughout the cell cycle. Thus, the increase in CAD CPSase activity cannot be attributed to an increase in autophosphorylation. Moreover, neither PKA nor MAP kinase phosphorylation affects the CPS-
ase activity in the absence of allosteric ligands. Thus, it is likely that the approximate 2-fold increase in CPSase activity is simply a consequence of the increase in the CAD concentration in the cell that must occur during each turn of the cell cycle. This interpretation is supported by assaying aspartate transcarbamoylase, an unregulated CAD activity, which was also found to increase 2-fold by the end of the cell cycle.

These changes in CPSase activity cannot explain the activation of the pathway that occurred during the S phase of the cell cycle. For example, between 6 and 10 h, where pyrimidine biosynthesis was up-regulated by 85%, the CPSase activity increased only 5%. However, there were appreciable changes (Fig. 5A, Table II) in the allosteric properties of CPSase. As the cells approached S phase, the activation of the enzyme by 200 μM PRPP increased about 2-fold, from 1240 to 2524%. In serum-starved cells, UTP (0.5 mM) inhibition of CPSase activity was 40%, but as the cells approached S phase, the effect of UTP was completely abolished. Both of these changes in allosteric regulation, which would be expected to increase the rate of pyrimidine biosynthesis, were fully or partially reversed by 14 h post-serum stimulation. Thus, the changes in the sensitivity of CPSase to PRPP and UTP closely paralleled the S phase and the activation of the pyrimidine biosynthetic pathway.

**Kinase Activities and CAD Phosphorylation State**—The MAP kinase activity (Fig. 6A) was lowest in early G1 and increased 15-fold during the first 4 h after serum stimulation. The activation of MAP kinase persisted for several hours and then began to decrease just before entry into the S phase of the cell cycle. The levels remained at ~50% of the maximum value up to 20 h. Despite high levels of MAP kinase activity during G1, the CAD phosphothreonine remained unchanged until just before S phase and then increased 2-fold. The phosphothreonine antibodies also detect phosphorylation of the single CAD autophosphorylation site (Thr-1037), and this site was found to be nearly fully phosphorylated throughout the cell cycle. Because there was a background phosphorylation of 1 mol of phosphothreonine, a 2-fold increase can be attributed to the incorporation of 1 mol of phosphate into the single CAD MAP kinase site (Thr-456). This interpretation was reinforced by the observation that exposure of cells 10 h post-serum stimulation (Fig. 6C), at which point the CAD MAP kinase site was 40% modified, to 20 μM PD98059 or UO126, two MEK (MAP kinase kinase) inhibitors, for 30 min resulted in a 35 and 40% reduction in phosphothreonine, respectively. These inhibitors had no effect on CAD phosphoserine. Similar results were obtained for cells harvested at other time points.

The PKA activity (Fig. 5B) was highest immediately after serum stimulation and began to decrease to 50% of the maximum value over the next 6 h. The inactivation of PKA was accompanied by a simultaneous decline in CAD Ser phosphorylation. Exposure of cells 2.5 h after serum stimulation to the PKA inhibitor, H89 (Fig. 6C), reduced the CAD serine phosphorylation by another 25%, with no effect on CAD phosphothreonine. The levels remained low until 16 h, at which point both the PKA activity and the PKA phosphorylation of CAD began to increase once again.

**Intracellular Localization of the Kinases and CAD**—For phosphorylation to occur, the kinase and its substrate CAD must be co-localized within the same intracellular compartment. Plasmids were constructed encoding fusion proteins that had the green or cyan fluorescent protein appended to the amino acids.
end of MAP kinase, the PKA catalytic subunit, the GLN-CPS domain, and CAD. The constructs were transiently transfected into BHK cells, and the intracellular location was visualized (Fig. 7) by fluorescent microscopy at 12-h intervals after transfection. Previous studies (49) show that the transfection procedure results in synchronization of the subpopulation of cells that have taken up the plasmid. MAP kinase was found to be primarily localized in the nucleus, although an appreciable amount was cytoplasmic throughout the time course. By contrast, PKA was initially localized in the nucleus but was subsequently translocated into the cytoplasmic compartment. Interestingly, a significant fraction of CAD, initially cytoplasmic, had migrated into the nucleus by 24 h. A similar distribution was observed for the GLN CPS domain, indicating that the putative nuclear targeting signals are located within this region of CAD. Immunofluorescence microscopy (not shown) showed that the high levels of cyclin E were expressed at 0 and 12 h, whereas the levels were much lower in the 24-h cultures. These results suggested that the 0- and 12-h samples represent G0/G1 and that by 24 h, the transfected subpopulation had entered the S phase of the cell cycle.

**DISCUSSION**

The results reported here are in agreement with previous studies of synchronously growing cells showing that the *de novo* pyrimidine biosynthetic pathway is activated just before S phase and subsequently down-regulated at the transition between S and G2 (Fig. 8). We found that the rate increased 1.9-fold from 1.0 pmol/min/10⁶ cells in G₁ to 1.85 pmol/min/10⁶ cells in mid-S phase. These rates are in good agreement with the rate of pyrimidine biosynthesis of 0.5 pmol/min/10⁶ cells measured in asynchronous cultures of the same cells during the first 24 h after passage but are significantly lower than the rate of 4.2 pmol/min/10⁶ cells measured in fully established, exponentially growing cells (18). The lower rate of pyrimidine biosynthesis after serum starvation can probably be attributed to the low intracellular concentration of the CPSase substrate, ATP.

The average values for the intracellular nucleotide triphosphates in mammalian cells, compiled by Traut in an exhaustive literature search (50), were found to be 567 ± 460, 278 ± 242, 3152 ± 1698, and 468 ± 224 μM for UTP, CTP, ATP, and GTP, respectively. The nucleotide triphosphate concentrations, measured by HPLC analysis of extracts of exponentially growing BHK cells (Table I), were for the most part within the expected range. It is noteworthy, that the nucleotide concentrations after stimulation of serum-deprived cells were appreciably lower that the values in exponentially growing cultures. Moreover, the nucleotide pools did not appreciably expand during the course of the cell cycle. Only modest increases were observed even after 20 h of post-serum stimulation (Table I). These results suggest that 1) the increased rate of pyrimidine biosynthesis nearly matches the rate of utilization of nucleotides for the synthesis of macromolecules during the S phase of the cell cycle, and 2) several cycles of cell division are apparently required before the nucleotide pools can be fully replenished after serum starvation. ATP, for example, was found to be 0.6 mM in serum-starved cells compared with a value of 2.9 mM obtained in fully established cultures. The low ATP concentration would be expected to result in an approximate 5-fold decrease in CPSase activity in serum-starved cells, in reasonable agreement with the Hill equation assuming a Kₘ of 3.4 mM and a Hill coefficient of 1.3, values measured (41) for purified CAD in the absence of allosteric effectors. Because the kinetic parameters are dependent on the concentration of effectors, the calculation is only approximate.
ment with the 7-fold decrease in the flux through the pyrimidine pathway compared with the rate in exponential cultures. As described above, the increase in pyrimidine biosynthesis during the S-phase of the cell cycle cannot be attributed to an increase in the CPSase activity measured in the absence of allosteric effectors or to autophosphorylation of CAD. The CAD autophosphorylation site was nearly completely phosphorylated in vitro, representing a constant phosphothreonine background throughout the cell cycle. However, previous studies show (18) that the CPSase activity of CAD, and consequently the rate of pyrimidine biosynthesis, is controlled by PKA- and MAP kinase-mediated phosphorylation of the CAD CPSase domain.

The activity of the pyrimidine biosynthetic pathway was up-regulated as the cells entered the S phase, reached a maximum at 11 h, and then decreased as the cells exited the S phase. The increase in the pyrimidine biosynthetic activity during S phase closely paralleled the response of CPSase to allosteric effectors. During S phase, UTP inhibition was abolished, and PRPP activation increased 2-fold. This altered response to allosteric effectors could readily account for the 1.9-fold activation of the pathway.

Similar changes in allosteric regulation have been observed in asynchronous cultures in response to MAP kinase phosphorylation (18). In synchronized BHK cells, the MAP kinase activity increased rapidly and peaked at 5 h but remained elevated for the duration of the cycle. However, the phosphorylation of the MAP kinase site of CAD did not correlate with the sensitivity of CAD to PRPP activation (32). Accordingly, a 1.7-fold decrease in the response to UTP and PRPP, allosteric effectors, could readily account for the 1.9-fold activation of the pathway. The phosphorylation and dephosphorylation of the regulatory sites on the CPSase domain of CAD alter the response to UTP and PRPP, allosteric effectors that control the flux through the pyrimidine pathway.

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