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# Chemically induced premature mitosis: Differential response in rodent and human cells and the relationship to cyclin B synthesis and p34$^{cdc2}$/cyclin B complex formation

**(premature chromosome condensation/DNA replication/okadaic acid/caffeine/protein kinase inhibitors)**

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**ABSTRACT** **Normal eukaryotic cells do not initiate mitosis until DNA replication has been completed. This requirement can be bypassed by exposing cells to certain chemicals. We report here that chemically induced premature mitosis is not readily achieved in all mammalian species. Although hamster cells underwent premature mitosis following treatment with caffeine, the protein phosphatase inhibitor okadaic acid, and the protein kinase inhibitors 2-aminopurine and 6-dimethylaminopurine, the mouse and human cells examined in this study displayed little or no response to any of these compounds. Differences in cell permeability or metabolism could not account for the species specificity of these drugs, because other biochemical and mitosis-promoting activities were apparent in human cells. Cell-type specificity can be explained, however, by the timing of cyclin B synthesis and p34$^{cdc2}$/cyclin B complex formation during the cell cycle. Synthesis of cyclin B and formation of a p34$^{cdc2}$/cyclin B complex, both of which are required for initiation of mitosis, were prevalent in hamster cells arrested in S phase but were absent or barely detectable in arrested human cells. In hamster cells, the hyperphosphorylated form of p34$^{cdc2}$ was complexed with cyclin B and underwent tyrosine dephosphorylation during caffeine-induced premature mitosis. These findings indicate that the onset of mitosis is regulated somewhat differently among mammalian cell types and that these differences affect the vulnerability of cells to drug-induced mitotic aberrations and cytogenetic damage.**

Cell cycle events are thought to be regulated by a series of interdependent biochemical steps, with the initiation of late events requiring the successful completion of those preceding them (1). When cells are arrested during S phase by chemical inhibitors or temperature-sensitive mutations, cells do not initiate mitosis until the inhibiting conditions are reversed and the cells are allowed to complete DNA replication. The biochemical events that couple mitotic onset to the completion of DNA replication remain unknown, but recently it has become possible to manipulate this regulatory pathway with certain chemicals and with genetic mutants.

Caffeine, the protein kinase inhibitors 2-aminopurine (2-AP) and 6-dimethylaminopurine (6-DMAP), and the protein phosphatase inhibitor okadaic acid have been shown to induce mitosis in hamster cells that are arrested in S phase by DNA synthesis inhibitors (2–5). Genetic studies in mammalian cells and in fungi have identified several genes that are important for the proper timing of mitosis. The temperature-sensitive mutants tsBN2 and bimE7, of Syrian hamster fibroblasts and *Aspergillus nidulans*, respectively, undergo mitosis at the restrictive temperature when cells are arrested in S phase (6, 7). In addition, when *cdc25* is overexpressed in a *wee*$^-$ background in *Schizosaccharomyces pombe*, mitosis is initiated before DNA replication is completed (8).

The onset of mitosis in all eukaryotic cells studied is controlled by a protein complex called maturation-promoting factor (MPF). MPF contains a serine/threonine protein kinase (p34$^{cdc2}$) and cyclin (9). An additional component of MPF, a 65-kDa protein dimer, has recently been identified in human mitotic cells (10). Microinjection experiments using an antibody to p34$^{cdc2}$ (11) and cell cycle studies with a mouse cell line containing a temperature-sensitive *cdc2* gene product (12) have shown that this kinase is essential for entry of mammalian cells into mitosis. The phosphorylation state of p34$^{cdc2}$ is cell cycle-regulated, with dephosphorylation leading to increased kinase activity during mitosis (13). The cyclins that were first discovered in sea urchin and clam embryos progressively increase in concentration as cells approach mitosis and rapidly degrade during the transition from metaphase to anaphase (14). Cyclins have now been identified in cells of numerous species, including humans (15–17). Cyclins undergo phosphorylation and are complexed with p34$^{cdc2}$ during mitosis, coincident with increased activity of this kinase, while cyclin degradation is associated with exit from mitosis and decreased kinase activity (17–19).

Okadaic acid, a potent tumor promoter and specific inhibitor of protein phosphatases 2a and 1 (20, 21), induces maturation when microinjected into *Xenopus* (22) and starfish (23, 24) oocytes and enhances MPF activation in *Xenopus* egg extracts (25) and in hamster fibroblasts (5). The action of okadaic acid upon MPF activation is indirect and apparently does not affect the phosphatases responsible for dephosphorylating p34$^{cdc2}$ at the onset of mitosis.

Here we show that in hamster cells several chemicals suppressed radiation-induced $G_2$ delay and uncoupled mitosis from the completion of DNA replication. In human cells these compounds overcame $G_2$ delay following DNA damage but failed to initiate mitosis prior to the completion of DNA replication. The cell specificity of these compounds for altering the temporal sequence of DNA replication and mitosis was related to cell-type differences in the timing of cyclin B synthesis and p34$^{cdc2}$/cyclin B complex formation during the cell cycle.

## MATERIALS AND METHODS

**Cell Culture and Metabolic Labeling.** Chinese hamster ovary (CHO) cells were grown in McCoy's medium containing 10% iron-supplemented calf serum (HyClone) and 4 mM glutamine. All other cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing the same additions. New cultures were started from frozen stocks every 4–6 weeks. Cells were mycoplasma-free based upon fluorescent

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Abbreviations: MPF, maturation-promoting factor; 2-AP, 2-aminopurine; 6-DMAP, 6-dimethylaminopurine.

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staining with Hoechst 33258. Arrest in S phase was achieved by addition of 2.5 mM hydroxyurea. Mitotic and premature mitotic cells were accumulated with nocodazole (0.25 μg/ml) and harvested by gentle physical detachment (26). Interphase cells were collected by treating monolayer cultures with 0.5 mM EDTA for 5–10 min. Cell populations were typically 90–95% pure as determined by chromosome preparations. Okadaic acid (from L. Levine), caffeine, 2-AP, and 6-DMAP (Sigma) were added to the medium at the appropriate concentrations. For metabolic labeling of proteins, cells were incubated with $^{32}P_i$ (40–400 μCi/ml; 1 Ci = 37 GBq) or [$^{35}$S]methionine (100 μCi/ml) in phosphate- or methionine-deficient DMEM containing 10% dialyzed calf serum.

**Quantification of Premature Mitosis.** The percentage of cells undergoing premature mitosis was determined by one of two methods. When drug treatments produced little or no toxicity, cells were fixed directly on the culture dishes in absolute methanol and stained for 10 min with Hoechst 33258 (1 μg/ml). Nocodazole (0.25 μg/ml) was used to accumulate cells at mitosis. Drug-induced cell toxicity and cytoskeletal changes sometimes resulted in cells either detaching from the culture dishes or displaying aberrant nuclear morphology that could possibly be confused with chromatin condensation. These effects were seen only after exposure to the higher doses of okadaic acid and 6-DMAP. Chromosome spreads were prepared in such cases to allow analysis of detached cells and to assure proper identification of premature mitotic events. Cells were swelled for 8–15 min (depending on cell type) in 75 mM KCl, fixed for 10 min in methanol/acetic acid (3:1, vol/vol), and dropped on wet slides before staining with Hoechst 33258 (1 μg/ml, 10 min). Premature mitosis was confirmed by the breakdown of the nuclear envelope and by the condensed and "pulverized" chromatin morphology indicative of cells in S phase that are forced to undergo premature chromatin condensation (27). For each method, at least 300 cells were examined at ×400 magnification by fluorescence microscopy to determine the percentage of cells having undergone premature mitosis. Mitotic delay in $G_2$ was induced by γ irradiation from a $^{60}$Co source. The percentage of mitotic cells was determined as above by fixing cells on the culture dishes and staining with Hoechst 33258. In this case, at least 1000 cells were examined for each time point.

**Immunoprecipitations, Electrophoresis, and Immunoblotting.** Antiserum raised against the C-terminal peptide (LDNQIKKM) of the mammalian p34$^{cdc2}$ protein was provided by P. Nurse (12). Human cyclin B antiserum was provided by J. Pines (17). Procedures for p34$^{cdc2}$ immunoprecipitations were as described (28). Control experiments used p34$^{cdc2}$ antiserum preincubated for 1 hr with the octapeptide LDNQIKKM (25 ng/μl of antiserum). Cyclin B immunoprecipitations employed the procedures of Gould and Hunter (29) but used the lysis buffer of Gautier *et al.* (28).

Protein samples were prepared in 2% SDS/5% 2-mercaptoethanol/10% glycerol/62.5 mM Tris, pH 6.8, and heated at 100°C for 5 min before electrophoresis (30). Protein concentrations were estimated (31) using γ-globulin as the standard. Radioactively labeled proteins were detected by autoradiography or fluorography of fixed and dried gels.

After electrophoresis, proteins to be immunoblotted were transferred to nitrocellulose in a tank blotting system (30 mA overnight followed by 60 mA for 1 hr in transfer buffer containing 25 mM Tris/192 mM glycine/20% methanol). Procedures used for p34$^{cdc2}$ immunoblotting were essentially as described (28). For phosphotyrosine immunoblotting, nitrocellulose was blocked for 2 hr with Tris-buffered saline (pH 7.5) containing 0.3% Tween-20 (TBST) and subsequently incubated overnight with anti-phosphotyrosine antiserum (1:1000 dilution in TBST) provided by L. Levine (32). Blots were washed three times in TBST, incubated with $^{125}$I-labeled protein A (0.5 μCi/ml in TBST), and washed four times in TBST. Phosphotyrosine-containing proteins and p34$^{cdc2}$ were detected by indirect autoradiography using an intensifying screen.

## RESULTS

**Insensitivity of Mouse and Human Cells to Chemically Induced Uncoupling of Mitosis from the Completion of DNA Replication.** Okadaic acid, caffeine, 2-AP, and 6-DMAP are able to uncouple mitosis from the completion of DNA replication in hamster cells. We wanted to determine whether the response to these chemicals was unique to hamster cells or was a general phenomenon seen in many mammalian species. Three human cell types (FS-2, normal foreskin fibroblast; HT1080, fibrosarcoma; HeLa, epithelioid carcinoma) as well as fibroblasts from two hamster species (BHK, Syrian hamsters; CHO, Chinese hamsters) and two mouse strains (A31, BALB/c mice; NIH 3T3, Swiss mice) were examined. Following arrest in S phase with hydroxyurea, cells were exposed to various concentrations of caffeine, 2-AP, 6-DMAP, and okadaic acid and examined for premature mitosis (Table 1). Hamster cells had a strong response to all chemicals tested. BHK cells showed a somewhat greater response than CHO cells at all drug concentrations. The greatest difference was seen with 6-DMAP. The toxicity of 5 mM 6-DMAP to CHO cells may account for the low percentage of cells entering mitosis prematurely. A slightly higher dose of 6-DMAP (10 mM) had been shown to adversely affect premature mitosis in BHK cells as well (4). The mouse and human cells that were tested displayed little or no response to any of the compounds. In addition to the cells examined in Table 1, several other rodent cell lines and primary cells and at least six additional human cell lines derived from various tissues were tested with 5 mM caffeine. Premature mitosis was seen only in hamster cells (data not shown). To eliminate trivial explanations of species specificity due to differences in chemical permeability or metabolism, we investigated other biochemical and mitosis-promoting effects of these compounds on hamster and human cells.

**Human Cells Are Sensitive to Chemicals That Abolish $G_2$ Delay.** Damage to cellular DNA often leads to a delay in the $G_2$ phase of the cell cycle (33), and caffeine is known to abolish this delay in both hamster and human cells (34–36). Suppression of DNA damage-induced $G_2$ delay is a form of premature mitosis, but this effect does not require an alter-

Table 1. Chemically induced premature mitosis

| Cell type | Drug (conc.) | % premature mitosis |
|---|---|---|
| Hamster | | |
| BHK | Caffeine (1, 2, 5 mM) | 53, 66, 67 |
| | 6-DMAP (1, 2, 5 mM) | 20, 37, 40 |
| | 2-AP (1, 2, 5 mM) | 3, 14, 34 |
| | Okadaic acid (0.5, 1, 2 μM) | 16, 35, 51 |
| CHO | Caffeine (1, 2, 5 mM) | <1, 4, 48 |
| | 6-DMAP (1, 2, 5 mM) | <1, 3, 2 |
| | 2-AP (1, 2, 5 mM) | <1, 1, 29 |
| Mouse (NIH 3T3, A31) | Caffeine (1, 2, 5 mM) | <1 |
| | 6-DMAP (1, 2, 5 mM) | <1 |
| | 2-AP (1, 2, 5 mM) | <1 |
| Human (HeLa, FS-2, HT1080) | Caffeine (1, 2, 5 mM) | <1 |
| | 6-DMAP (1, 2, 5 mM) | <1 |
| | 2-AP (1, 2, 5 mM) | <1 |
| | Okadaic acid* (0.5, 1, 2 μM) | <1 |

Cells were arrested in S phase for 3–5 hr with 2.5 mM hydroxyurea and then treated with caffeine, 6-DMAP, or 2-AP for 8 hr in the presence of nocodazole (0.25 μg/ml). Okadaic acid treatment was 6 hr.
*HeLa cells were the only human cells treated with okadaic acid.

ation in the temporal sequence of DNA replication and mitosis. We wanted to determine whether 2-AP and 6-DMAP were also capable of suppressing $G_2$ delay. A positive response in both hamster and human cells would demonstrate both the bioavailability of the chemicals to both species and the ability of these compounds to alter a regulatory pathway controlling mitotic onset that does not require the uncoupling of mitosis from the completion of DNA replication.

Asynchronously growing cultures of BHK and HeLa cells received 250 rads (1 rad = 0.01 Gy) of $\gamma$ radiation and then were either left untreated or immediately exposed to caffeine (2 mM), 2-AP (5 mM), or 6-DMAP (1 mM). The percentage of mitotic cells in the cultures (mitotic index) was monitored to determine the extent of mitotic delay under these conditions (Fig. 1). After irradiation, BHK and HeLa cells rapidly underwent a $G_2$ arrest that was reversed without further treatment within 4–5 hr. Caffeine, 2-AP, and 6-DMAP suppressed this mitotic delay in both hamster and human cells. These compounds had little or no effect on the mitotic index of nonirradiated cells during this period (data not shown). Prolonged exposure, however, did reduce the mitotic index in all cases. The doses chosen for these experiments were the minimum dose of each chemical that produced a nearly complete suppression of mitotic delay in BHK cells. These same concentrations were equally effective in HeLa cells (Fig. 1*B*). HeLa cells are, therefore, as sensitive as BHK cells to chemically induced premature mitosis when arrested in $G_2$ phase but are insensitive when arrested in S phase.

**Okadaic Acid Inhibits Phosphatase Activity in Hamster and Human Cells.** The effect of okadaic acid on radiation-induced mitotic delay could not be readily determined because this chemical, unlike caffeine, 2-AP, and 6-DMAP, induced premature mitosis in exponentially growing as well as cell cycle-arrested cells. To help eliminate the possibility that the species specificity of okadaic acid-induced premature mitosis was due to differences in the efficiency of phosphatase inhibition, we examined the ability of okadaic acid to increase the level of intracellular protein phosphorylation in BHK and HeLa cells. Treatment of cells with this chemical is known to produce a rapid increase in the level of protein phosphorylation without affecting the specific radioactivity of intracellular ATP or the ATP/ADP ratio (21).

BHK and HeLa cells were prelabeled with $^{32}P_i$ for 1 hr followed by addition of 2 $\mu$M okadaic acid or diluent for additional periods of time. Increases in protein phosphorylation were seen in both BHK and HeLa cells within 1 hr after the addition of okadaic acid (Fig. 2). It is unlikely, therefore, that the insensitivity of human cells to okadaic acid-induced premature mitosis is due to an inability of the compound to alter the state of intracellular protein phosphorylation.

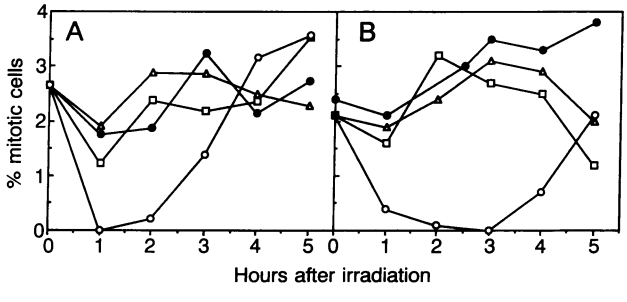


FIG. 1. Suppression of radiation-induced mitotic delay in BHK and HeLa cells. Asynchronous cultures of BHK (*A*) and HeLa (*B*) cells were exposed to 250 rads of $\gamma$ radiation. Immediately after irradiation, cells either remained untreated (○) or were treated with caffeine (●, 2 mM), 2-AP (□, 5 mM), or 6-DMAP (△, 1 mM). At the indicated times, cells were fixed and stained, and the mitotic index was determined.

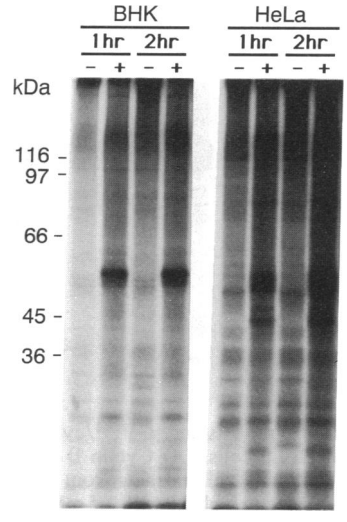


FIG. 2. Effect of okadaic acid on protein phosphorylation in BHK and HeLa cells. Cells were prelabeled for 1 hr with $^{32}P_i$ (40 $\mu$Ci/ml). Labeling was continued for 1 or 2 hr in the presence of either 0.3% dimethyl sulfoxide diluent (−) or 2 $\mu$M okadaic acid (+). Phosphoproteins from whole cell extracts were separated in a 10% polyacrylamide gel and detected by autoradiography. Equal numbers of cells were used for okadaic acid-treated and untreated groups.

**Cyclin B Synthesis and p34$^{cdc2}$/Cyclin B Complex Formation in Hamster and Human Cells.** Activation of p34$^{cdc2}$ is dependent upon the formation of a complex with cyclin B. The presence or absence of this complex during S-phase arrest could explain cell-type differences in susceptibility to chemically induced uncoupling of mitosis from the completion of DNA replication. To address this possibility, BHK and HeLa cells were arrested during S phase with hydroxyurea, cellular proteins were metabolically labeled with [$^{35}$S]methionine, and the presence of cyclin B and p34$^{cdc2}$/cyclin B complexes was examined with antibodies against p34$^{cdc2}$ and cyclin B. Immunoprecipitations using cyclin B antiserum showed that in BHK cells that were arrested in S phase, but not in arrested HeLa cells, cyclin B was actively synthesized and was complexed with p34$^{cdc2}$ (Fig. 3*A*). Identical results were obtained with immunoprecipitations using p34$^{cdc2}$ antiserum (Fig. 3*B*). The signal strength for p34$^{cdc2}$ in BHK and HeLa cells was equalized in Fig. 3*B* to eliminate the possibility that the absence of p34$^{cdc2}$ in cyclin B immunoprecipitations of HeLa cells was due to reduced p34$^{cdc2}$ synthesis.

Labeling asynchronous cultures with [$^{35}$S]methionine induces $G_2$ delay due to DNA damage, and this delay can be suppressed by exposing cells to caffeine. To allow an approximation of cyclin B synthesis in $G_2$ and mitotic cells, asynchronous BHK and HeLa cells were labeled with [$^{35}$S]methionine and treated with caffeine. The resulting mitotic cells were collected and analyzed. Under these conditions, cyclin B was synthesized and found to be complexed with p34$^{cdc2}$ in both BHK and HeLa cells (Fig. 3*A*). In BHK cells, cyclin B synthesis during arrest in S phase was roughly equivalent to that seen in cells rescued from mitotic delay by caffeine treatment.

In S-phase-arrested BHK cells, cyclin B antibody coprecipitated only the hyperphosphorylated form of p34$^{cdc2}$ (Fig. 3*A*). This form of the kinase can be identified by its slower migration in polyacrylamide gels. The hyperphosphorylated form of p34$^{cdc2}$ existed almost exclusively in a complex with cyclin B, since partial clearing of cyclin B from BHK extracts removed essentially all of the hyperphosphorylated form from subsequent immunoprecipitations with p34$^{cdc2}$ antiserum (Fig. 3*B*). No hyperphosphorylated form of p34$^{cdc2}$ was

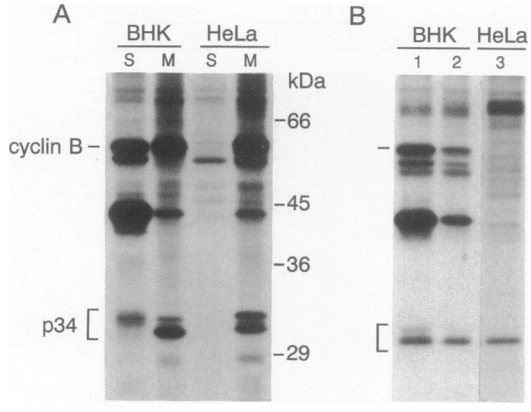


FIG. 3. Cyclin B synthesis and p34$^{cdc2}$/cyclin B complex formation in BHK and HeLa cells. (*A*) Cyclin B immunoprecipitations were performed on whole cell extracts from BHK and HeLa cells that were either arrested in S phase with 2.5 mM hydroxyurea for 6–8 hr and labeled with [$^{35}$S]methionine (100 $\mu$Ci/ml) during the final 3 hr (S) or rescued from mitotic delay induced by [$^{35}$S]methionine labeling (100 $\mu$Ci/ml for 8 hr) by simultaneous treatment with 5 mM caffeine (M). Rescued mitotic cells were accumulated in the presence of nocodazole (0.25 $\mu$g/ml) and collected by physical detachment. (*B*) p34$^{cdc2}$ immunoprecipitations were performed on whole cell extracts from BHK and HeLa cells that were arrested and labeled in S phase, as described above. Lane 2 is identical to lane 1 except that immunoprecipitation of p34$^{cdc2}$ was preceded by partial clearing of cyclin B protein by preincubation for 1 hr with cyclin B antiserum and subsequent removal with protein A-Sepharose. Immunoprecipitations were performed with equal amounts of acid precipitable radioactivity ($10^7$ cpm for *A*; $3 \times 10^7$ cpm for *B*). Proteins were separated in 12% polyacrylamide gels and detected by fluorography. To achieve equivalent p34$^{cdc2}$ signal intensity for BHK and HeLa cells in *B*, lane 3 was exposed to film twice as long as lanes 1 and 2.

detected in S-phase-arrested HeLa cells (Fig. 3*B*), consistent with the lack of cyclin B synthesis.

Using extracts derived from *Xenopus* eggs, Solomon *et al.* (37) found that the binding of cyclin to p34$^{cdc2}$ induced tyrosine and threonine phosphorylation of the previously unphosphorylated enzyme. One would therefore expect that the hyperphosphorylated form of p34$^{cdc2}$ in S-phase-arrested BHK cells would contain phosphotyrosine, whereas no phosphotyrosine would be evident in p34$^{cdc2}$ from S-phase-arrested HeLa cells. Anti-p34$^{cdc2}$ immunoblotting showed that asynchronous cultures of BHK and HeLa cells contained a slower-migrating form of p34$^{cdc2}$ (Fig. 4*A*). Immunoprecipitates of p34$^{cdc2}$ that were immunoblotted with p34$^{cdc2}$ and phosphotyrosine antisera showed that this form of the kinase was also present in S-phase-arrested BHK cells but was absent from arrested HeLa cells (Fig. 4*B*). The phosphotyrosine immunoblot confirmed that the slower-migrating form of p34$^{cdc2}$ from BHK cells contained phosphotyrosine (Fig. 4*C*). Phosphotyrosine was not detectable in p34$^{cdc2}$ of S-phase-arrested HeLa cells.

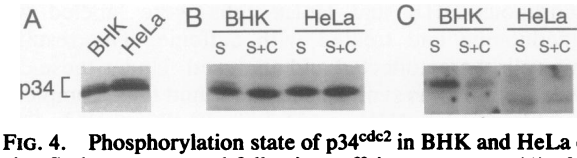


FIG. 4. Phosphorylation state of p34$^{cdc2}$ in BHK and HeLa cells during S-phase arrest and following caffeine treatment. (*A*) p34$^{cdc2}$ immunoblot of whole cell extracts from asynchronous cultures of BHK and HeLa cells. Equal amounts of protein (100 $\mu$g) were added to both lanes. (*B* and *C*) p34$^{cdc2}$ was immunoprecipitated from cell extracts of BHK and HeLa cells that were arrested in S phase for ≈12 hr and received either no additional treatment (S) or 5 mM caffeine treatment (S+C) during the final 6–8 hr. Both caffeine-treated and untreated groups were exposed to nocodazole (0.25 $\mu$g/ml) during the final 6–8 hr to accumulate cells undergoing premature mitosis. Equal numbers of cells were used for caffeine-treated and untreated groups. Immunoprecipitated proteins were divided equally into two parts, separated in a 10% polyacrylamide gel, and immunoblotted with antibodies against p34$^{cdc2}$ (*B*) and phosphotyrosine (*C*).

**Caffeine-Induced Dephosphorylation of p34$^{cdc2}$ in BHK Cells.** Antibodies against p34$^{cdc2}$ and phosphotyrosine were used to determine whether caffeine induced the mitotic form of p34$^{cdc2}$ during premature mitosis of S-phase-arrested BHK cells. $^{32}$P-labeled whole cell extracts of interphase, mitotic, and caffeine-induced premature mitotic cells were immunoprecipitated with anti-p34$^{cdc2}$ antibody (Fig. 5). Distinct interphase and mitotic forms of p34$^{cdc2}$ can be discerned, and caffeine-induced premature mitotic cells contained only the mitotic form of the enzyme, which is underphosphorylated and migrates faster in polyacrylamide gels. Immunoblots of p34$^{cdc2}$ immunoprecipitates confirmed that caffeine treatment reduced the abundance of the hyperphosphorylated form of p34$^{cdc2}$ (Fig. 4*B*) and induced a simultaneous loss in tyrosine phosphorylation (Fig. 4*C*). As expected, no changes in electrophoretic mobility or tyrosine phosphorylation were detected in similarly treated HeLa cells. p34$^{cdc2}$ was expressed at equivalent levels in BHK and HeLa cells as well as in caffeine-treated and untreated cells (Fig. 4*B*). Absence of tyrosine phosphorylation cannot, therefore, be an artifact due to lower expression of the protein. The mitotic form of p34$^{cdc2}$ was still labeled with $^{32}$P (Fig. 5). Morla *et al.* (13) reported that p34$^{cdc2}$ was completely dephosphorylated in NIH 3T3 cells that were arrested in mitosis. The present study in BHK cells, as well as recent work using *Xenopus* egg extracts (37), found p34$^{cdc2}$ to be partially phosphorylated during mitosis. It is unclear whether this discrepancy is the result of differences in cell type or in experimental design.

## DISCUSSION

Mammalian cells contain several checkpoints in the cell cycle that monitor both external and internal conditions critical for controlled cell growth (1). The timing of mitotic onset is affected by at least two of these checkpoints. Under normal circumstances, cells will not initiate mitosis in the presence of unreplicated or damaged DNA. A limited number of chemicals can cause mammalian cells to bypass regulatory mechanisms governing mitotic onset. Caffeine can prevent DNA damage-induced mitotic delay in numerous mammalian species, including humans (34–36). 2-AP and 6-DMAP were shown in this report to have similar effects. It is known that caffeine, 2-AP, 6-DMAP, and okadaic acid can induce mitosis in hamster cells that contain incompletely replicated genomes

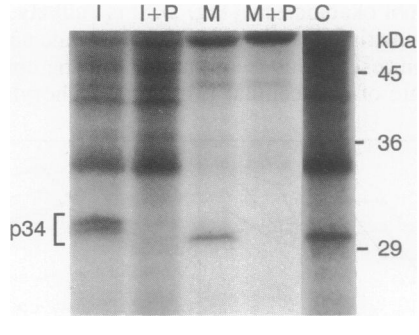


FIG. 5. Immunoprecipitation of $^{32}$P-labeled extracts from BHK cells with p34$^{cdc2}$ antiserum. Interphase (I), mitotic (M), and caffeine-induced premature mitotic (C) cells were labeled for 4 hr with $^{32}P_i$ (400 $\mu$Ci/ml). Mitotic cells were accumulated for 6 hr in nocodazole (0.25 $\mu$g/ml) prior to labeling, and premature mitotic cells were obtained by treating labeled interphase cells with caffeine (5 mM) for 6 hr. Immunoprecipitations were performed with equal amounts of radioactivity ($2 \times 10^6$ cpm), and proteins were detected by indirect autoradiography using an intensifying screen. The peptide LDNQIKKM was used to compete with endogenous p34$^{cdc2}$ in interphase (I+P) and mitotic (M+P) extracts.

(2, 4, 5). This latter activity has been reported for caffeine in cell-free extracts of *Xenopus* eggs as well (38). Okadaic acid was found to induce maturation in *Xenopus* (22) and starfish (23, 24) oocytes as well as to potentiate MPF activation *in vitro* in *Xenopus* egg extracts (25). Considering the extensive interspecies activity of these compounds, it was unexpected to find such marked specificity within mammalian cells for uncoupling mitosis from the completion of DNA replication.

This differential response by mammalian cells suggested that the hamster cells examined in this study, but not the mouse or human cells, accumulated factors required for premature mitotic onset during arrest in S phase. Our finding that cyclin B was actively synthesized and that $p34^{cdc2}$ and cyclin B formed a complex in BHK cells, but not HeLa cells, that were arrested in S phase is consistent with this hypothesis. The formation of this complex, which is required for mitosis, and the concomitant tyrosine phosphorylation of $p34^{cdc2}$ are intermediate steps in the activation pathway of MPF (37). The absence of these events in S-phase-arrested HeLa cells can account for the insensitivity of these cells to chemically induced premature mitosis. This insensitivity may be beneficial to human cells, since premature mitosis causes extensive cytogenetic damage and nuclear fragmentation (2, 3, 39).

The ability of caffeine, 2-AP, and 6-DMAP to induce mitosis in both hamster and human cells that were delayed in $G_2$ by DNA damage indicated that the factors necessary for chemically induced mitosis eventually accumulated in human cells, but did so later in the cell cycle, sometime during the transition from S to $G_2$. This S-to-$G_2$ transition is the time when HeLa cells initiate cyclin B synthesis (17), implicating cyclin B as a limiting factor for drug-induced mitosis. This requirement for cyclin B is further supported by the inability of caffeine and okadaic acid to induce mitosis in BHK cells that are synchronized in $G_1$, when little cyclin B is present (2, 5). As mentioned previously, caffeine and okadaic acid advance meiosis and mitosis in oocyte and egg systems. Extensive synthesis of cyclin B in interphase eggs and stockpiling of cyclin B in oocytes (40, 41) probably account for the responsiveness of these cells.

The mechanisms by which caffeine, 2-AP, 6-DMAP, and okadaic acid ultimately induce premature mitosis are not well understood. Changes in the normal state of protein phosphorylation appear to be very important. Okadaic acid is a specific inhibitor of protein phosphatases 2A and 1 (20, 21, 42), and caffeine, 2-AP, and 6-DMAP can inhibit protein kinase activity *in vitro* (43–45). It is interesting that inhibitors of protein phosphatases and protein kinases can produce a similar effect. This apparent discrepancy is likely due to the indirect action of these compounds. Caffeine and the protein kinase inhibitors 2-AP and 6-DMAP require new protein synthesis for induction of premature mitosis (2, 4). Although okadaic acid does not have such a requirement (5), okadaic acid must be activating MPF by an indirect method, because activation is accompanied by dephosphorylation of $p34^{cdc2}$ at threonine and tyrosine residues (13). The key regulatory molecules altered by these drugs, as well as the mechanisms by which mammalian cells control the timing of cyclin B synthesis and $p34^{cdc2}$/cyclin B complex formation, remain unknown. It is clear from the present studies, however, that these chemicals act at a time in the cell cycle after the formation of a $p34^{cdc2}$/cyclin B complex but prior to tyrosine dephosphorylation of $p34^{cdc2}$. The timing of complex formation is a critical factor in determining susceptibility of cells to chemically induced uncoupling of mitosis from the completion of DNA replication.

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