Effect of benzo(a)pyrene and mitomycine C on HeLa cell division cycle

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ABSTRACT : Recently, there has been significant progress in understanding the control process of the cell division cycle. To investigate the influence of toxic substances on the cell cycle, the effect of benzo(a)pyrene (BAP) and mitomycine C (MMC) on synchronized HeLa cells was analyzed during the cell cycle. To synchronize the HeLa cells, 10^6 cells were grown for 1 day and then treated with 1 mM hydroxyurea for 14 h. The arrested cells were then allowed to proceed through their cell cycle by removing the hydroxyurea and resupplying a fresh medium. The arrested cells in the G1/S transition then proceeded to the S phase after 4 h, the G2/M phase after 8h, and the G1 phase after 12 h, subsequent to the resupply of a fresh medium. In the untreated HeLa cells, the $p34^{cdc2}$ kinase activity, measured using a $p34^{cdc2}$ specific peptide, peaked after 8h (G2/M) and then declined after 12 h (G1). However, treatment with 30 µM BAP delayed the peak of the $p34^{cdc2}$ kinase activity. The amount of $p34^{cdc2}$ remained unchanged in the untreated, BAP-, and MMC-treated cells throughout the cell cycle. The cyclin B level peaked after 8 h in the untreated cells, yet peaked after 10-12 h in the BAP-treated cells. There was no significant change in the cyclin B level in the MMC-treated cells.

Keywords : $p34^{cdc2}$ protein kinase, cyclin B, cell division cycle, HeLa cells, benzo(a)pyrene, mitomycin C.

Introduction

It is generally evident that various, if not all, toxicants exert many of their carcinogenic actions by either directly or indirectly disturbing the cell division cycle. The cell division cycle is controlled by growth regulators, such as cyclins, growth factors, and kinases, functioning either positively or negatively. As such, the loss of cell cycle control leads to altered cell growth, genetic instability and gene mutation. Recently, knowledge of the cellular and molecular basis of cell growth and division has progressed significantly.

Eukaryotic cells in proliferation, either G1/S or G2/M, appear to involve a protein kinase first identified as cdc2 (Nurse and Bisset, 1981). A mutation of the cdc2 gene gives rise to cell division cycle arrest in the fission yeast Schizosaccharomyces pombe (reviewed by Nurse, 1990). The human homologue, $p34^{cdc2}$ (CDK1), is a protein serine/threonine kinase (Lee and Nurse, 1987) that is catalytically active when complexed with cyclins. Cyclin is a family of proteins that are cyclically synthesized and catastrophically degraded at specific points in the cell division cycle (Draetta and Beach, 1988; Draetta et al., 1989). The activation of $p34^{cdc2}$ kinase is essential for the induction of S phase and M phase (Lee and Nurse, 1987; Draetta and Beach, 1988).

Cyclins, essential components of the maturation promoting factor (MPF), were first described as early embryonic proteins in marine invertebrates (Evans et al., 1983). Although many cyclins have been since cloned and identified, no enzymatic activity has been attributed to cyclin B, while $p34^{cdc2}$ is known to include protein kinase activity that is essential for the initiation of the M phase. Functional cyclin B is required to start the M phase and activate $p34^{cdc2}$. As such, $p34^{cdc2}$ is the catalytic subunit of the MPF heterodimer, and cyclin B has a regulatory function.

Benzo(a)pyrene (BAP), an environmental carcinogenic polyaromatic hydrocarbon (PAH), is metabolized by cytochrome P450 into reactive diol epoxides that covalently bind to DNA bases (Harvey, 1991; Osborne, 1987). In addition to activating BAP into DNA binding metabolites, substantial amounts of reactive oxygen species, such as

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superoxide anion radicals, singlet oxygen, and catalase-inhibitable hydrogen peroxide are also generated (Frenkel *et al.*, 1988; Kagan *et al.*, 1988). However, the effect of the DNA-binding and damage caused by these metabolites on the cell cycle is still unknown.

Current knowledge on growth regulator behavior in the cell division cycle upon contact with toxicants is limited. Accordingly, the current study investigated the effect of toxicants on the cell cycle in "a synchronized HeLa cell model". The synchronized HeLa cell culture was expected to provide more detailed information on the effect of toxicants on the level of p34^{cdc2} kinase relating with cyclin B.

Materials and Methods

Cells

The HeLa cells were grown in 100mm culture dishes in Dulbeccos modified Eagles medium (DMEM) supplemented with penicillin and streptomycin, and 10% (v/v) fetal bovine serum at 37°C. For the cell division cycle experiments, the HeLa cells were exposed to 1 mM hydroxyurea (Sigma) for 14-16 h to arrest the cells at the G1/S transition (Ashihara & Baserga 1979). The arrested cells were then allowed to proceed through their cell cycle by removing the hydroxyurea and resupplying a fresh medium (Yu *et al.*, 1991). To harvest the HeLa cells during the cell cycle, the cells were washed 3 times with ice-cold phosphate buffered saline (PBS), scraped off the plate using a cell scraper, lysed by the addition of a lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 10 mM EDTA and 1 mM phenylmethylsulfonylfluoride), and then centrifuged at 10,000 g for 1 min. The supernatants were stored at -70°C.

Flow cytometry

A flow cytometric analysis was performed as previously described by Buchkovich *et al.* (1989). The cells (2×10^6) were washed and resuspended in 0.5 ml of ice cold PBS, then fixed by the gradual addition of 95% ethanol (-20°C) while vortexing.

MTT assay

MTT ((1-{4,5-dimethylthiazol-2yl}-3,5-diphenylformazan. Sigma) was dissolved in PBS to make a 5 mg/ml solution and then filtered for the purpose of sterilization and to remove any insoluble residues. The MTT solution was added to each well of a 96-well plate containing 5×10^4 HeLa cells/well in 100 μl of the culture medium. The plate was incubated at 37°C in a 5% CO$_2$ humidified incubator.

After 2 h, 100 μl of 10% SDS in 0.01 N HCl was added to each well, then the plate was incubated for a further 2 h. Aliquots was taken from each well and their absorbance measured at 570 nm.

Protein kinase assay

The protein kinase activity was assayed as previously described (Carroll and Marshak, 1989). The peptide ADAQHATPPKKKRKVEDPKDF (CSH 103), based on LTag (Marshak *et al.*, 1991), was used as the p34^{cdc2} specific substrate. The p34^{cdc2} activity was determined by measuring the incorporation of [^{32}PO$_4$] into the p34^{cdc2} specific peptide substrate. 5μl samples were incubated in a final volume of 30 μl at 37°C for 20 min in the presence of a p34^{cdc2} buffer (20 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$. 1 mM EGTA, 1 mM DTT) along with their respective peptide (1 mM) in the presence of 100 μM [γ-^{32}P]-ATP (1000-3000 cpm/pmol, Amersham corp.). The reactions were initiated by the addition of radioactive ATP. The assays using the peptide were then stopped by the addition of trichloroacetic acid to a final concentration of 10% (v/v), then the phosphate incorporation was determined based on adsorption to phosphocellulose paper, according to previously described protocols (Kuenzel & Krebs, 1985; Marshak & Carroll, 1991).

Gel electrophoresis and Immunoblotting

The electrophoresis was performed on 10% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulfate using the buffer system of Laemmli (1970). The proteins were transferred electrophoretically to nitrocellulose (Towbin *et al.*, 1979 & 1984). The nitrocellulose was then blocked with 3% bovine serum albumin (BSA) (w/v) in PBS and incubated with either a rabbit anti- p34^{cdc2} antibody (Oncogene Science) or monoclonal anti-cyclin B antibody (Oncogene Science) at the desired dilutions in the same buffer, An alkaline phosphate-labeled goat anti-rabbit IgG or goat anti-mouse IgG (Sigma) was used as the secondary antibody. The nitrocellulose paper was developed as previously described (Harlow and Lane, 1988).

Results

Cytotoxic effect of BAP and MMC

Th cytotoxic effect of BAP ranged from 480 μM to 7.5 μM in the HeLa cells, as evaluated by the MTT analysis (Fig. 1). 30 μM of BAP (open squares) exhibited 15%, 18%, and 45% cytotoxicity after 24 h, 48 h, and 72 h, respectively. The highest concentration of 480 μM resulted in the complete inhibition of cell growth. The cytotoxic effect of MMC
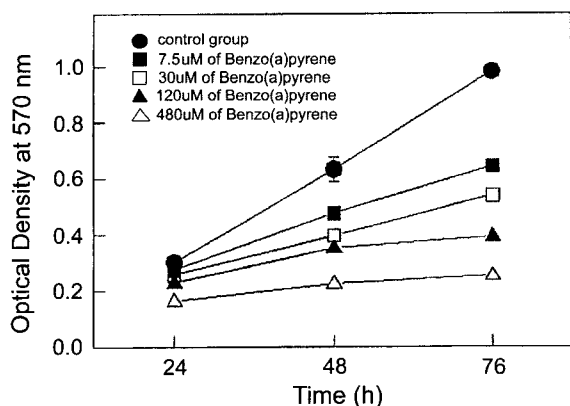
Fig. 1. Effect of benzo(a)pyrene on viability of HeLa cells.
HeLa cells (5×10^5/well) grown in a 96-well plate were treated with 7.5 µM, 120µM, and 480 µM of benzo(a)pyrene for 24-76 h at 37°C, in a 5% CO_2 humidified incubator. Samples were collected every 24 h and processed for an MTT assay. Closed circles (untreated), closed squares (7.5 µM), open squares (30 µM), closed triangles (120 µM), open triangles (480 µM of benzo(a)pyrene).
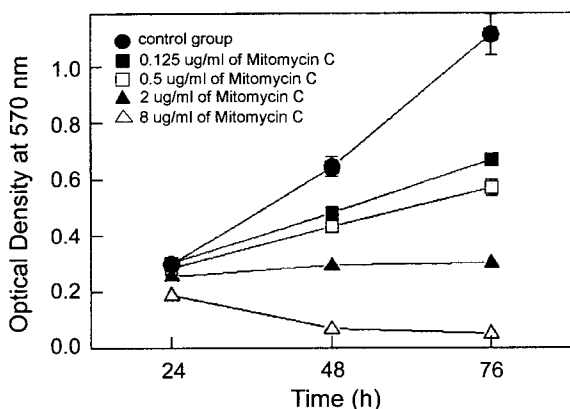

Fig. 2. Effect of mitomycin C on viability of HeLa cells.
HeLa cells (5×10^5/well) grown in a 96-well plate were treated with 0.125 µg/ml, 0.5 µg/ml, 2 µg/ml, and 8 µg/ml of mitomycin C for 24-76 h at 37°C in a 5% CO_2 humidified incubator. Samples were collected every 24 h and processed for an MTT assay. Closed circles (untreated), closed squares (0.125 µg/ml), open squares (0.5 µg/ml), closed triangles (2 µg/ml), open triangles (8 µg/ml of mitomycin C).

ranged from 0.125 µg to 8 µg, also as evaluated by the MTT analysis (Fig. 2). 0.5 µg of MMC exhibited 12% cytotoxicity after 24 h, 33% after 48 h, and 48% after 72 h. The highest concentration of 8 µg resulted in a significant inhibition of cell growth.

Synchronization and progression of cell cycle

The HeLa cells that were arrested with hydroxyurea in the G1/S cycle were then released by the removal of hydroxyurea enabling them to progress through the cell
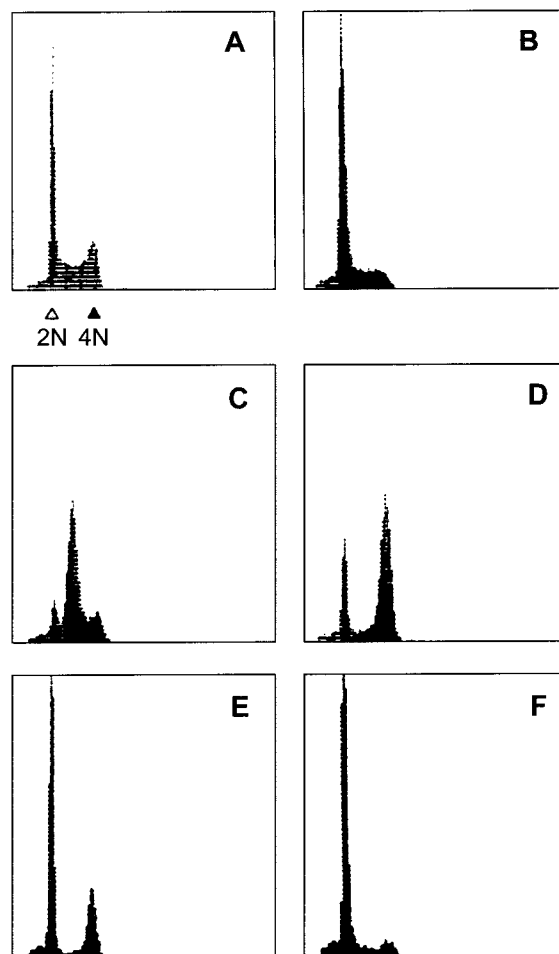
Fig. 3. FACS analysis of hydroxyurea arrest released HeLa cells. Asynchronous HeLa cells arrested by hydroxyurea for 14 h were washed and incubated with a new medium. Samples were collected every 4 h and processed for an FACS analysis. The open and closed triangles mark the 2N and 4N DNA content, respectively. A, asynchronous HeLa cells; B, 0 h; C, 4 h; D, 8h; E, 12 h; F, 16 h after release.

cycle (Fig. 3). The asynchronous HeLa cells consisted of 53.5% in G1, 16.5% in S, and 24.9% in G2/M, while the hydroxyurea-arrested HeLa cells consisted of 70% in G1, 13.3% in S, and 12.3% in G2/M (Fig. 4A). Four hours after the removal of hydroxyurea, the cells predominantly shifted into the S phase of the cell cycle and consisted of 18.8% in G1, 54.4% in S, and 21.3% in G2/M (Fig. 4B). After 8 h, the cells entered the mitotic phase and consisted of 23.3% in G1, 11.4% in S, and 57.8% in G2/M (Fig. 4C). After 12 h, most of the cells reached the G1 phase of the cell cycle and consisted of 63.6% in G1, 5.7% in S, and 24.9% in G2/M (Fig. 4D). After 16 h, the cells consisted of 74.5% in G1, 7.6% in M, and 10.7% in G2/M.
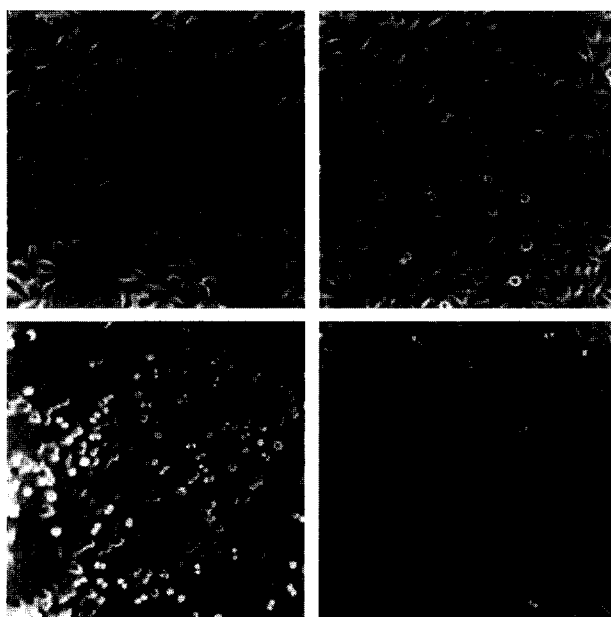
Fig. 4. Morphological appearance of synchronized HeLa cells. Asynchronous HeLa cells arrested by hydroxyurea for 14 h were washed and incubated with a new medium. The development of the cultures was recorded using an inverted phase contrast microscope (Olympus). The composite picture shown here contains photographs taken A, 0 h; B, 4 h; C, 8 h; D, 12 h, after the release. All photographs were taken with a 10x objective. Many cells in the mitotic phase can be seen in C.
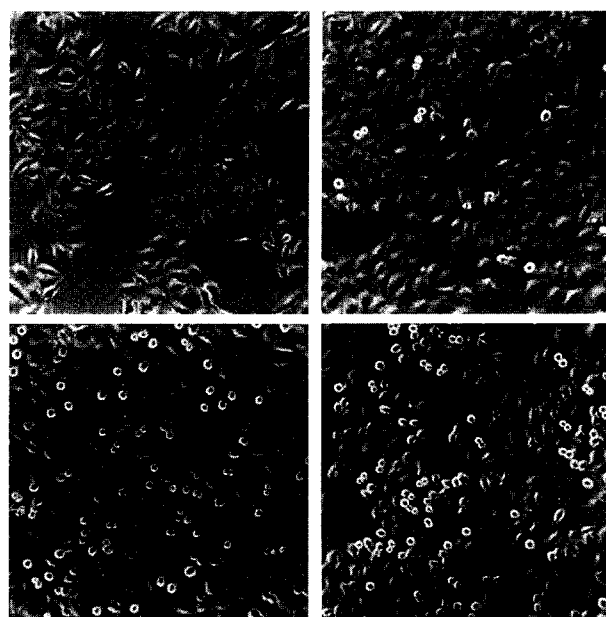

Fig. 5. Morphological appearance of 30 μM of benzo(a)pyrene-treated HeLa cells after hydroxyurea release. Asynchronous HeLa cells arrested by hydroxyurea for 14 h were washed and incubated with a medium containing 30 μM of benzo(a)pyrene. The development of the cultures was recorded using an inverted phase contrast microscope (Olympus). A, 0 h; B, 4 h; C, 8 h; D, 12 h, after the release. Many cells in the mitotic phase can be seen in C and D.

Effect of BAP and MMC on cell division

The effect of BAP and MMC on the cell division was observed, and the cells in division were counted. The synchronized HeLa cells were reacted with 30 μM BAP or 0.5 μg/ml of MMC for 12 h. The cells in division exhibiting a round shape after detaching from the plate surface were counted under a microscope. The percentages of cells in division are shown in Table 1. The untreated cells exhibited their normal cell division with 52.8% of the cells in division after 8 h (M phase). The HeLa cells treated with BAP exhibited a delayed cell division with only 38% after

Table 1. Percentage of cells in division

Time (h)	0	4	8	12
	%	%	%	%
Untreated	0	7.7	52.8	17
30 μM of BAP	0	7.0	38.0	26.9
0.5 μg/ml of MMC	0	3.5	0.7	2.0

Asynchronous HeLa cells arrested by hydroxyurea for 14 h were washed and treated with a medium containing 30 μM of benzo (a)pyrene or 0.5 μg/ml of mitomycin C. The cells in division showing a round shape after detaching from the plate surface were counted under a microscope. The percentage of cells in division was calculated.

8 h and 26.9% after 12 h (Fig. 5 C &D, Table 1). In contrast, the MMC-treated cells exhibited a complete inhibition of cell division and showed no round cells (Fig. 6 C&D, and Table 1).

Effect of BAP and MMC on p34^{cdc2} kinase activity

The effect of BAP and MMC on the cell division cycle was analyzed by measuring the p34^{cdc2} kinase activity in the synchronized HeLa cells. The p34^{cdc2} kinase activity reached a maximum level after 8 h and then decreased after 12-18 h (Fig. 7), corresponding to the G2/M and G1 phases of the cell cycle, as shown in Fig. 1. Although the untreated HeLa cells exhibited a typical pattern of p34^{cdc2} kinase activity change (Fig. 8, closed squares), the kinase activity in the 30 μM BAP-treated cells was prolonged by 12 h (Fig. 8). Meanwhile, the kinase activity in the 0.5 μg/ml of MMC-treated HeLa cells was inhibited throughout the cell cycle.

Effect of BAP and MMC on p34^{cdc2} kinase and cyclin B level

The levels of p34^{cdc2} kinase were measured to analyze the effect of BAP and MMC on the kinase activity during
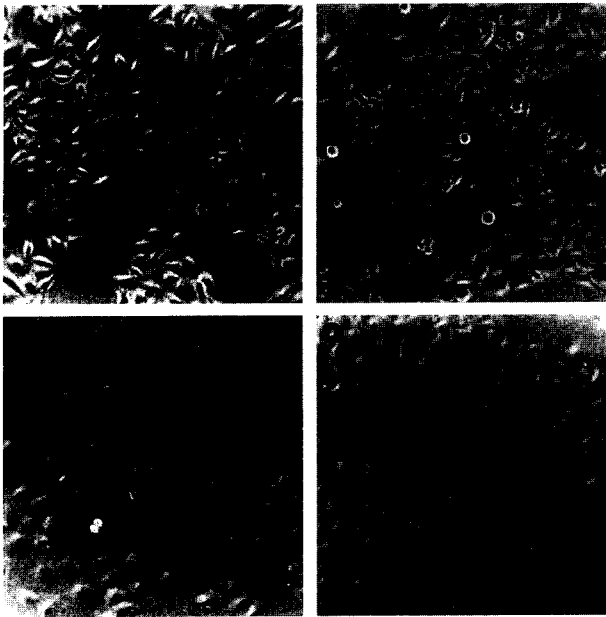
Fig. 6. Morphological appearance of 0.5 μg/ml mitomycin C-treated HeLa cells after hydroxyurea arrest release.

Asynchronous HeLa cells arrested by hydroxyurea for 14 h were washed and incubated with a medium containing 0.5 μg/ml of mitomycin C. The development of the cultures was recorded using an inverted phase contrast microscope (Olympus). The composite picture shown here contains photographs taken A, 0h; B, 4 h; C, 8h; D, 12 h, after the release. All photographs were taken with a 10x objective. Only a few cells in the mitotic phase can be seen in C and D.
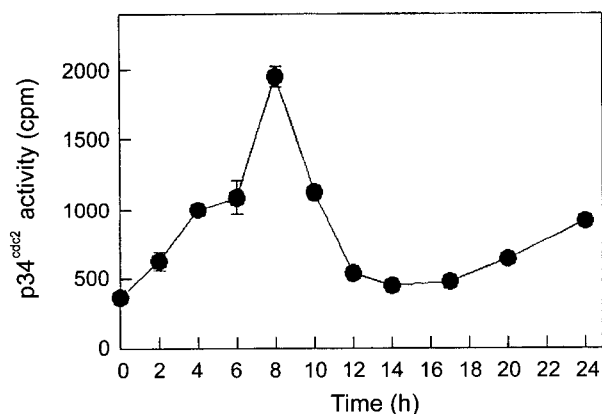
Fig. 7. $p34^{cdc2}$ kinase activity of hydroxyurea arrest released HeLa cells. The HeLa cell growth was arrested in a medium containing hydroxyurea for 14 h. After washing the cells three times with PBS and washing the sample plates, a fresh medium was added at time 0. Sample cells were scraped every 2 h. Cell extracts were prepared and quantitated to ensure an equal amount of protein for the kinase assay. The $P34^{cdc2}$ kinase activity was measured against a $p34^{cdc2}$ specific peptide substrate.
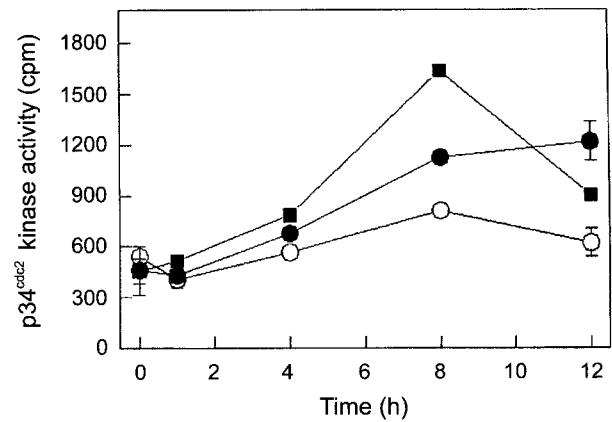

Fig. 8. Effect of benzo(a)pyrene and mitomycin C on $p34^{cdc2}$ activity. Hydroxyurea arrested HeLa cells were released at time 0 by the addition of a fresh medium, or a medium containing 30 μM benzo(a)pyrene or 0.5 μg/ml of mitomycin. Samples were prepared after 0, 1, 4, 8, and 12 h and processed for a $p34^{cdc2}$ kinase activity assay. Closed squares, untreated; closed circles, 30 μM of benzo(a)pyrene; open circles, 0.5 μg/ml of mitomycin C.

the cell cycle. The changes in the $p34^{cdc2}$ kinase activity were not found to be due to a decrease or increase in the $p34^{cdc2}$ protein level, instead the amount of $p34^{cdc2}$ remained relatively constant, as shown by the immunoblot analysis (Fig. 9). The protein levels of $p34^{cdc2}$ in the untreated HeLa cells remained constant during the cell cycle. Furthermore, the levels of $p34^{cdc2}$ in the BAP and MMC-treated cells also stayed nearly the same throughout the cell cycle.

Changes in $p34^{cdc2}$ kinase activity are already known to be associated with cyclin B. Therefore, the effect of BAP and MMC on the synchronized HeLa cells was examined by measuring the protein levels of cyclin B using an cyclin B monoclonal antibody (Fig. 10). The protein levels of cyclin B in the untreated HeLa cells peaked after 6 h, corresponding to the early G2/M phase of the cell cycle. However, the BAP-treated cells exhibited their maximum cyclin B level after 10 h, corresponding to the $p34^{cdc2}$ kinase activity shown in Figure 6. Interestingly, the MMC-treated cells did not exhibit any cyclin B throughout the cell cycle.
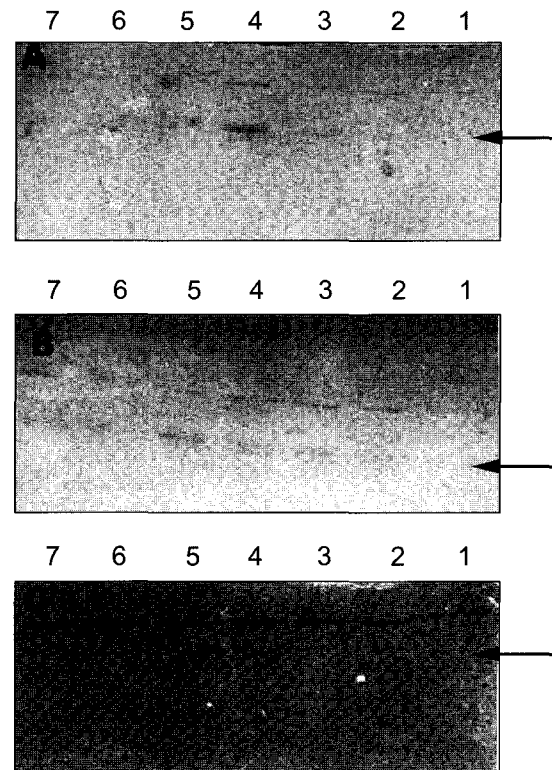
Discussion

The culture system adopted in the current study provided a detailed investigation on the effect of toxicants on the cell cycle. To analyze the components involved in the progression of the cell cycle, a synchronized HeLa cell culture was utilized. HeLa cells synchronized by hydroxyurea treatment were arrested in the G1/S transition after 12-14 h
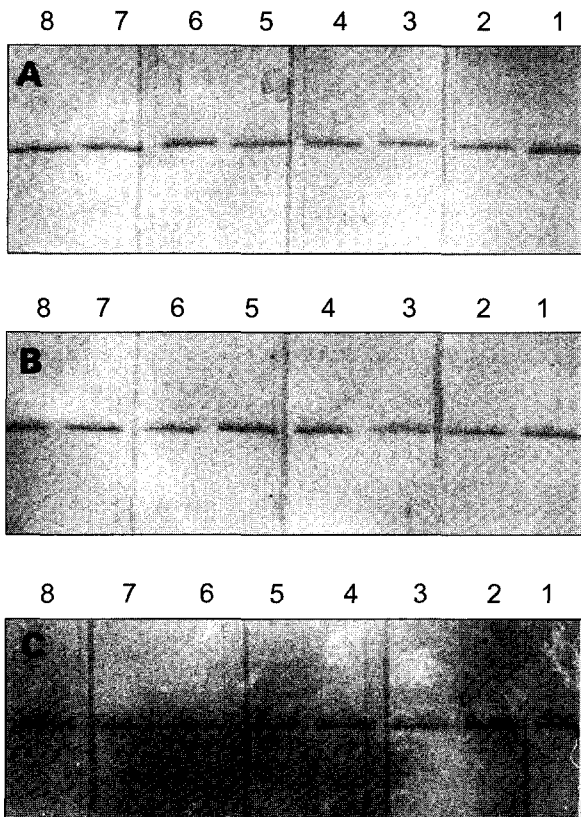
Fig. 9. Immunoblotting of anti-p34^{cdc2} antibody with synchronized HeLa extracts.

HeLa cell extracts prepared at designated times were subjected to electrophoresis on a 12.5% polyacrylamide gel, transferred to nitrocellulose, and probed with an anti p34^{cdc2} antibody. A, untreated HeLa cells; B, 30 μM of benzo(a)pyrene-treated HeLa cells; C, 0.5 μg/ml of MMC-treated HeLa cells. Lane 1, 0 h; lane 2, 0.5 h; lane 3 1 h; lane 4, 2 h; lane 5, 4 h; lane 6, 8h; lane 7, 12 h; lane 8, 24 h.

Fig. 10. Immunoblotting of anti-cyclin B antibody with synchronized HeLa extracts.

HeLa cell extracts prepared at designated times were subjected to electrophoresis on a 12.5% polyacrylamide gel, transferred to nitrocellulose, and probed with an anti cyclin B antibody. A, untreated HeLa cells; B, 30 μM of benzo(a)pyrene-treated HeLa cells; C, 0.5 μg/ml of MMC-treated HeLa cells. Lane 1, 0 h; lane 2, 0.5 h; lane 3 1 h; lane 4, 2 h; lane 5, 4 h; lane 6, 8 h; lane 7, 12 h; lane 8, 24 h.

of treatment with 1 mM hydroxyurea, as also found in previous publications. The removal of the drug and supply of a fresh medium then released the HeLa cells and allowed them to continue their cell division cycle. Using this synchronized culture, the effects of toxicants were examined as regards their role in the cell cycle on a molecular level. 30 μM of BAP produced a delayed appearance of the maximum p34^{cdc2} kinase activity and low cytotoxicity in the HeLa cells. While the HeLa cells treated with 1.5 μM of MMC showed a significant inhibition of p34^{cdc2} kinase acitivity and similar low cytotoxicity as found with 30 μM of BAP. Accordingly, these results indicate that the inhibition of p34^{cdc2} kinase activity was neither due to a low protein level of cyclin B nor p34^{cdc2}. In the BAP-treated HeLa cells, the level of cyclin B reached a peak after 10 h when the p34^{cdc2} kinase activity was at its maximum level. However, in the MMC-treated

HeLa cells, a very low level of cyclin B was observed throughout the HeLa cell cycle, indicating the complete inhibition of p34^{cdc2} kinase activity in these cells.

In the present study, BAP, which is known to cause DNA adduct formation, did not apparently affect p34^{cdc2} on a transcriptional level, yet did affect the transcription of cyclin B. Although, it is also possible that BAP may have affected p34^{cdc2} through post-translational modification. It is also unclear whether BAP produces a p34^{cdc2} or cyclin B protein adduct that can hinder the association of cyclin B and p34^{cdc2}, which is essential to p34^{cdc2} kinase activity. The DNA adducts formed by BAP may also have affected the transcription of cyclin B, thereby delaying the cyclin B formation. However, since the HeLa cells used in the current study do not have an active mixed functional oxidase system like hepatocytes, the delaying of the cyclin B synthesis was on a somewhat basal level. Accordingly, if hepatocytes were used, the delaying of the cell cycle may

be more apparent. A similar effect was previously seen in serum-starvation-synchronized Swiss 3T3 fibroblast cells (unpublished observation). MMC, known to cause DNA cross-linking, also did not affect the p34^{cdc2} level, yet did affect the formation of cyclin B. The DNA cross-linking caused by MMC may have completely blocked the transcription of cyclin B, thereby explaining the absence of cyclin B in the western blot.

Further analyses of the protein adduct formation by BAP need to be performed to understand the delaying of the cell cycle due to the inhibition of cyclin B formation, plus the role of BAP in transcriptional inhibition also needs to be investigated.

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