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Simian Virus 40 Prevents Activation of M-Phase-Promoting Factor during Lytic Infection

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Simian virus 40 (SV40) infection stimulates confluent cultures of monkey kidney cells into successive rounds of cellular DNA synthesis without intervening mitosis. As an initial step in defining the mechanisms responsible for viral inhibition of mitosis, M-phase-promoting factor (MPF) was examined in SV40-infected CV-1 cells passing from G_2 phase into a second S phase. MPF is a serine-threonine protein kinase that is essential for mitosis in eukaryotic cells. In SV40-infected cells exiting G_2 phase, there was a reduced amount of MPF-associated H1 kinase activity relative to that of uninfected cells passing through mitosis. Both subunits of MPF, cyclin B and the $p34^{cdc2}$ catalytic subunit, were present and in a complex in infected cells. In uninfected cultures, passage through mitosis was associated with the dephosphorylation of the $p34^{cdc2}$ subunit, which is characteristic of MPF activation. In contrast, the $p34^{cdc2}$ subunit remained in the tyrosine-phosphorylated, inactive form in SV40-infected cells passing from G_2 phase into a second S phase. These results suggest that although the MPF complex is assembled and modified normally, SV40 interferes with pathways leading to MPF activation.

Simian virus 40 (SV40) infection of permissive (24, 25), semipermissive (20, 30, 40), and nonpermissive (16, 23, 35) cells results in the emergence of tetraploid populations. The induction of tetraploidy is most efficient in the permissive (simian) system, in which viral DNA replication is accompanied by the continued stimulation of cellular DNA synthesis in greater than 80% of the population and the accumulation of a greater than G_2 (>G_2) amount of DNA within 2 days postinfection (24, 25). An observation consistent with the induction of successive S phases is that SV40-infected CV-1 cells do not show any morphological evidence of mitosis (11). SV40 T antigen is necessary and sufficient for the initial stimulation of G_1-phase cells into DNA synthesis (12, 37, 44, 47). Induction of DNA synthesis appears to be influenced by at least three functions of T antigen, including pRB binding; a function in the C-terminal half of T antigen, which may be p53 binding; and a novel function found in the DNA-binding domain (5). Stimulation of monkey kidney cells into a second S phase is dependent on a function of T antigen distinct from those required for the stimulation of G_1-phase cells into S phase (8).

The induction of consecutive S phases without mitosis would require that SV40 uncouple the normal pathways linking the completion of S phase to mitosis. The best-characterized component of the mitotic pathway, M-phase-promoting factor (MPF), is required for mitosis (6, 13, 39, 46) and acts through phosphorylation of multiple substrates. A number of chromosomal and cytoskeletal proteins, transcription factors, and protein kinases are candidate MPF substrates (32), which may be involved in the initiation and progression of mitosis. In vertebrates, inactive MPF is detected during G_2 phase as a complex between cyclin B and the hyperphosphorylated $p34^{cdc2}$ catalytic subunit (6, 18). Phosphate groups at T-14 and Y-15 are inhibitory for kinase activity (19, 33), whereas phosphorylation of T-161 is necessary for kinase activity (43). Y-15 is phosphorylated by the tyrosine kinase, Wee1 (29, 36), and dephosphorylated by Cdc25 phosphatase (22, 41). Although the kinase responsible for phosphorylation of T-14 has not been identified, it can be dephosphorylated by Cdc25 (41). The inactive form of MPF persists until late G_2 phase when the dephosphorylation of T-14 and Y-15 results in the activation of MPF kinase and the onset of mitosis.

The absence of mitosis in SV40-infected CV1 cells suggests that SV40 is interfering either with the normal pathways leading up to MPF activation or with events following MPF activation. To locate the step(s) in the mitotic pathway that is blocked by SV40, MPF activity was examined in SV40-infected cells passing from G_2 phase into a second S phase. Reduced MPF activity in infected cells would suggest that SV40 was interfering with a step in the pathway leading up to MPF activation. Alternatively, mitotic levels of MPF activity would suggest that MPF was activated normally but that the function of MPF in initiating mitosis was diverted by viral infection.

MATERIALS AND METHODS

Virus and cells. CV-1 cells are an African green monkey kidney cell line (ATCC CCL70) obtained from American Type Culture Collection. Cultures were maintained in modified Eagle's medium supplemented with 2× amino acids and vitamins, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5% fetal bovine serum (FBS). Cultures were split 1:4 and used between passages 30 and 40. SV40 strain RH911 was grown and plaque assayed on CV-1 cells (25). CV-1 cells and virus stocks were mycoplasma free (25).

CV-1 cultures at confluence for 2 to 4 days were infected with SV40 at 100 PFU per cell for 1 h at 37°C. Cultures were then refed with minimal essential medium–1% FBS. Uninfected confluent CV-1 cells were stimulated to cycle by trypsinization and replating at 1:2 in minimal essential medium–10% FBS. SV40-infected and uninfected cultures were synchronized by a late-G_1 mimosine blockade (48). Eight hours after infection or replating, mimosine (Sigma; 10 mM in PBS) was added to the culture media to a final concentration

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of 400 μM. After 16 h, mimosine-containing medium was removed and replaced by fresh medium without mimosine.

Flow cytometric analysis (FCM). Cultures were harvested by trypsinization, fixed in 90% methanol, and stored at −20°C. Cell suspensions were stained for DNA content with propidium iodide and for SV40 T antigen with Pab101 as previously described (21). Analysis was carried out on a Cytofluorograf IIs model 50 H-H and 2151 data analysis system (Ortho Diagnostics) in conjunction with a model 532 Omnichrome air-cooled argon laser (excitation at 488 nm, 20 mW of power) as described previously (21, 24, 25); 10,000 cells were analyzed per sample.

Mitotic index determinations. Culture supernatants were pooled with cell suspensions obtained by trypsinization of cell monolayers. At least 5.0×10^5 cells were washed in cold phosphate-buffered saline (PBS), pelleted, resuspended in distilled water-PBS (3:1) and incubated at room temperature for 10 min. Samples were gently pelleted, fixed in acetic acid-methanol (1:3) at 10^6 cells per ml, and stored at −20°C. After 15 to 20 μl of sample was dropped onto a cold, wet slide, the slides were dried and 500 to 1,000 cells were examined by phase-contrast microscopy to determine the percentage of cells containing condensed chromosomes.

Isolation of mitotic cells. T150 flasks of logarithmically growing cells were trypsinized and replated (1:1) in minimal essential medium plus 10% FBS. Six to 8 h after replating, colcemid was added to a final concentration of 0.05 μg/ml (stock of 10 μg/ml in PBS). Mitotic shakes were performed after 12 to 16 h and 20 to 24 h of colcemid block.

$p13^{suc1}$-Sepharose binding reactions. $p13^{suc1}$ was purified from *Escherichia coli* BL21(DE3)lysS (a gift of B. Ducommun), transformed with plasmid RK172 as previously described (1), and bound to CNBr-activated Sepharose 4B (Pharmacia), according to the manufacturer's instructions.

Extracts of SV40-infected and uninfected cells were prepared by first washing pooled supernatant cells and trypsinized monolayer cells with PBS containing the following protease and phosphatase inhibitors; 50 mM NaF, 0.1 mM sodium orthovanadate, 1 μg of leupeptin per ml, 10 μg of soybean trypsin inhibitor per ml, 1 μg of aprotinin per ml, and 0.1 mM phenylmethylsulfonyl fluoride. Cells were then lysed in EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40) plus inhibitors (3), at 10^7 cells per ml for 5 min on ice with occasional vortexing, and then pelleted at 10,000 × *g* for 10 min at 4°C; the supernatant was retained. $p13^{suc1}$-Sepharose beads were washed once with NET-N (20 mM Tris [pH 8.0], 100 mM NaCl, 1.0 mM EDTA, 0.5% Nonidet P-40) plus inhibitors (3), just prior to addition of lysate. For each reaction mixture, 40 μl of 25% $p13^{suc1}$-Sepharose slurry was gently pelleted through 500 μl of NET-N and then resuspended in 500 μl of NET-N. Lysate (500 μl) was added to the washed, resuspended beads, and the mixture was rocked for 2 h at 4°C. The beads were then gently pelleted and washed four times with NET-N plus inhibitors and either used immediately or quick-frozen in liquid nitrogen and stored at −70°C.

Immunoprecipitation. Plates were placed on ice, and supernatant cells from media and PBS washes were pelleted and combined with the lysed, adherent cells which were removed from the plates by scraping in lysis buffer (50 mM Tris [pH 8.0], 250 mM NaCl, 0.5% Nonidet P-40, plus protease and phosphatase inhibitors as described above). Cells in lysis buffer (3×10^7 cells per ml) were incubated for 5 min on ice with occasional vortexing and then pelleted at 10,000 × *g* for 10 min at 4°C. Lysate (100 μl) was reacted with 1 μg of monoclonal anti-human cyclin B1 antibody (Pharmingen) or with 1 μl of rabbit anti-human cyclin B (a gift of T. Hunter) at 4°C for 1 h with constant shaking. Antigen complexed with mouse monoclonal antibody was immobilized by shaking for 1 h at 4°C with protein A-agarose beads which had previously been reacted with rabbit anti-mouse immunoglobulin G1. Antigen complexed with rabbit antibody was immobilized directly on protein A-agarose. Beads and their associated antibody-antigen complexes were collected by gentle centrifugation, washed four times with cold NET-N plus inhibitors, used immediately or quick-frozen in liquid nitrogen, and stored at −70°C.

Immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gels were transferred to Immobilon, reacted with antibodies, and developed as previously described (9). Primary antibody dilutions were as follows: anti-$p34^{cdc2}$ antibody, G6, (a gift of G. Draetta), 1:3,000; monoclonal anti-human cyclin B1 (Pharmingen), 0.5 μg/ml; and monoclonal anti-phosphotyrosine (Oncogene Science), 10 μg/ml. Secondary antibodies conjugated to alkaline phosphatase (Fisher Scientific) were diluted as follows: goat anti-rabbit, 1:3,000, and goat anti-mouse, 1:1,000. Quantitative analysis of band intensity was performed on a Bio Image system (Millipore).

H1 kinase assays. $p13^{suc1}$-Sepharose precipitates were washed once with kinase wash buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 μM cold ATP, plus inhibitors) and resuspended in 100 μl of kinase reaction mixture (kinase wash buffer plus 0.083 mg of histone H1 [Sigma] per ml and 2.5 μCi of [γ-^{32}P]ATP per reaction). Samples were incubated for 5 min at 30°C with gentle agitation, and reactions were terminated by the addition of 15 μl of 1× SDS sample buffer (0.0625 M Tris-HCl [pH 6.8], 2.0% SDS, 10.0% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue). Samples were mixed well and immediately analyzed or frozen at −20°C. Immunoprecipitated samples were treated in the same manner, except that the reaction was in 50 μl of kinase buffer containing 5.0 μCi of [γ-^{32}P]ATP for 10 min at 30°C and terminated with 50 μl of 2× SDS sample buffer. Samples were analyzed on SDS–10% polyacrylamide gels and then autoradiographed. Histone H1 phosphorylation presented in Fig. 2 was quantified with a Beta-scope analysis system (Beta-gen).

RESULTS

Cdk activity does not increase in SV40-infected CV-1 cells exiting G_2 phase. In normally cycling cells exiting G_2 and entering M phase, there is a marked increase in cyclin-dependent kinase (Cdk) activity resulting primarily from the activity of $p34^{cdc2}$-cyclin B. To examine Cdk activity in SV40-infected and uninfected CV-1 cells leaving G_2 phase, synchronous cultures were obtained by releasing the cells from a late-G_1-phase block imposed by mimosine. Cell cycle progression was monitored by FCM quantification of DNA content.

Confluent CV-1 cells were either infected with SV40 or stimulated into the cell cycle by trypsinization and replating. Mimosine was then included in the media between 8 and 24 h after infection or replating. Greater than 80% of the cells in the mimosine-blocked uninfected (Fig. 1A) and infected (Fig. 1B) cultures were in G_1 phase. After release from mimosine, infected and uninfected cells moved synchronously, and at similar rates, through S and G_2 phases. In the infected cultures the cells moved into $>G_2$ between 10 and 16 h postrelease, while at the same time in uninfected cultures the cells cycled back into G_1 phase. Since FCM analysis does not distinguish G_2-phase cells from M-phase cells, the mitotic indexes of cultures parallel to those in Fig. 1A and B were also measured. In the uninfected cultures (Fig. 1C), increasing numbers of cells with condensed chromosomes were seen between 10 and

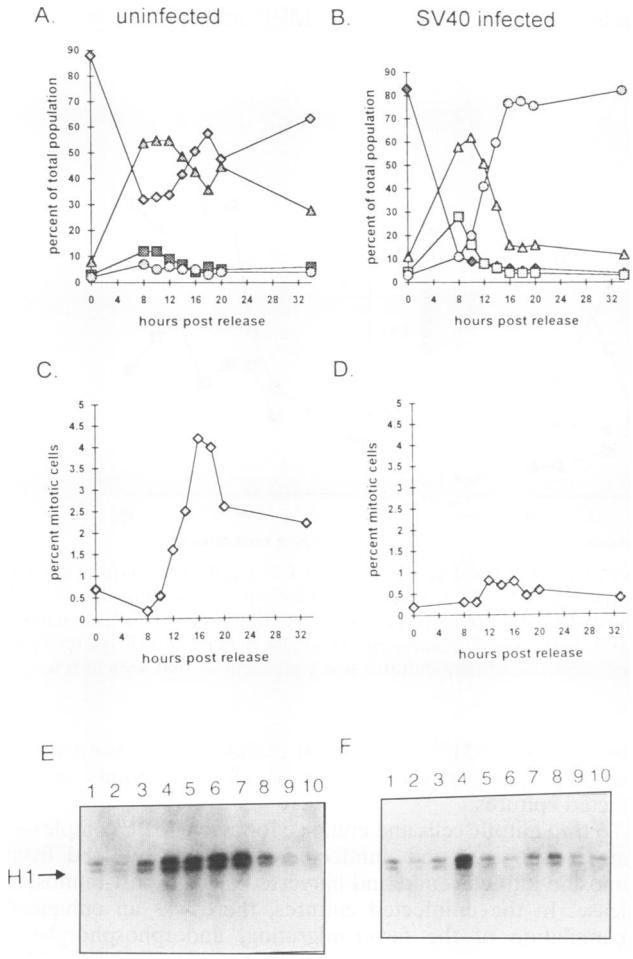

FIG. 1. Cell cycle progression and $p13^{suc1}$-Sepharose-precipitated H1 kinase activity of uninfected and SV40-infected CV-1 cells. Confluent CV-1 cells were trypsinized and replated at 1:2 (A, C, and E) or infected with SV40 at 100 PFU per cell (B, D, and F). Eight hours postinfection or replating, 400 μM mimosine was added. After 16 h, media were replaced with fresh media without mimosine. Samples were harvested at indicated times post-mimosine release. (A and B) Cells were fixed in 90% methanol and stained with propidium iodide for FCM analysis of DNA content. G_1 phase, diamond; S phase, square; G_2 phase, triangle; $>G_2$ phase, circle. (C and D) Cells were swollen in hypotonic buffer and fixed in acetic acid-methanol (1:3) for mitotic index determination. (E and F) $p13^{suc1}$-Sepharose-precipitated H1 kinase activity from cell lysates of confluent cells (lanes 1) and cells released from mimosine for 0 h (lanes 2), 8 h (lanes 3), 10 h (lanes 4), 12 h (lanes 5), 14 h (lanes 6), 16 h (lanes 7), 18 h (lanes 8), 20 h (lanes 9), and 34 h (lanes 10). Autoradiograph of SDS–10% PAGE. Infected cultures were greater than 90% T antigen positive at the time of mimosine release.

16 h post-mimosine release, whereas the mitotic index remained low throughout the mimosine release of the infected cultures (Fig. 1D).

To examine the effect of SV40 infection on Cdk activity in cells bypassing mitosis and traversing from G_2 to $>G_2$, $p13^{suc1}$-Sepharose-precipitated H1 kinase activity was measured on cultures parallel to those described above. Several Cdks, including Cdc2, Cdk2, and Cdk3, are known to bind $p13^{suc1}$. Both infected and uninfected cultures showed an increase in kinase activity at 10 h post-mimosine release. In the uninfected cultures there was continuous kinase activity over the next 6 h (Fig. 1E), which corresponded with the increasing mitotic index of the culture (Fig. 1C). The kinase activity of the uninfected cultures declined by 18 to 20 h postrelease, which corresponded with the population cycling back to G_1 (Fig. 1A). In contrast, the $p13^{suc1}$-Sepharose-precipitated H1 kinase activity in the infected cultures (Fig. 1F) declined by 12 h post-mimosine release and remained low, with minor fluctuations as the cells progressed from G_2 to $>G_2$ phase.

To determine whether the increase in $p13^{suc1}$-Sepharose-precipitated H1 kinase activity seen in the infected culture at 10 h post-mimosine release was due to MPF activity of uninfected mitotic cells in the cultures, infected cells were released from mimosine in the presence and absence of colcemid. Identical peaks of p13-associated H1 kinase activity were found at 10 h post-mimosine release in infected cultures grown with or without colcemid (not shown). Since colcemid blocks cells in prometaphase of mitosis, the failure of colcemid to alter the peak of activity in the infected cultures indicates that the peak of kinase activity at 10 h post-mimosine release was not due to mitosis of uninfected cells.

Reduced MPF activation in SV40-infected CV-1 cells exiting G_2 phase. The low levels of Cdk activity, as measured by $p13^{suc1}$-Sepharose binding, in SV40-infected cells traversing the border from G_2 to $>G_2$ phase suggested that MPF was not activated in infected cells leaving G_2 phase. To measure MPF activity more specifically, anti-cyclin B immunoprecipitations were performed over the course of a mimosine release of SV40-infected and uninfected cells. Confluent CV-1 cells were either SV40 infected or trypsinized and replated, blocked with mimosine, and released as described for Fig. 1. The MPF activity of uninfected cells moving through mitosis reached a level six- to sevenfold greater than that found in confluent or late-G_1-phase cultures (Fig. 2A and C). However, in SV40-infected cells progressing from G_2 to $>G_2$, MPF activity was increased threefold over that seen in confluent and late-G_1 cultures (Fig. 2B and C).

Cyclin B-associated $p34^{cdc2}$ is not dephosphorylated in SV40-infected CV-1 cells exiting G_2 phase. In vertebrate cells, the $p34^{cdc2}$ subunit of inactive MPF, found in late-S- and G_2-phase cells, is phosphorylated at T-14, Y-15, and T-161 (18). Activation of MPF and the initiation of mitosis is dependent on the loss of phosphates at T-14 and Y-15. To examine the phosphorylated state of $p34^{cdc2}$ in cyclin B complexes from SV40-infected cells traversing $G_2/>G_2$, anti-cyclin B immunoprecipitates were prepared from cultures parallel to those described in the legend to Fig. 2 and immunoblotted with antibody specific for $p34^{cdc2}$. On the basis of the previous finding that phosphorylation retards the migration of $p34^{cdc2}$ in SDS-polyacrylamide gel electrophoresis (PAGE), it was possible to monitor the degree of $p34^{cdc2}$ phosphorylation by its gel mobility. In the uninfected cultures, a population of cells progressed through G_2 phase between 12 and 14 h postrelease, passed through mitosis, and cycled back to G_1 by 16 to 20 h (Fig. 2A). The cyclin B-associated $p34^{cdc2}$ increased over the confluent level by 8 h post-mimosine release, continued to increase until 14 h postrelease (2.5-fold increase over levels at 8 h), and then decreased slightly at 18 to 20 h as the cells cycled back to G_1 (Fig. 3A). The $p34^{cdc2}$ coimmunoprecipitated from the uninfected culture at 8 h post-mimosine release was primarily the more slowly migrating, phosphorylated form of the protein (Fig. 3A, lane 4), but passage of the cells through M phase, between 12 and 18 h postrelease, was associated with the appearance of the underphosphorylated form (Fig. 3A, lanes 6 to 9).

In the infected cultures, cells progressed through G_2 phase at a rate similar to that of uninfected cultures and accumulated

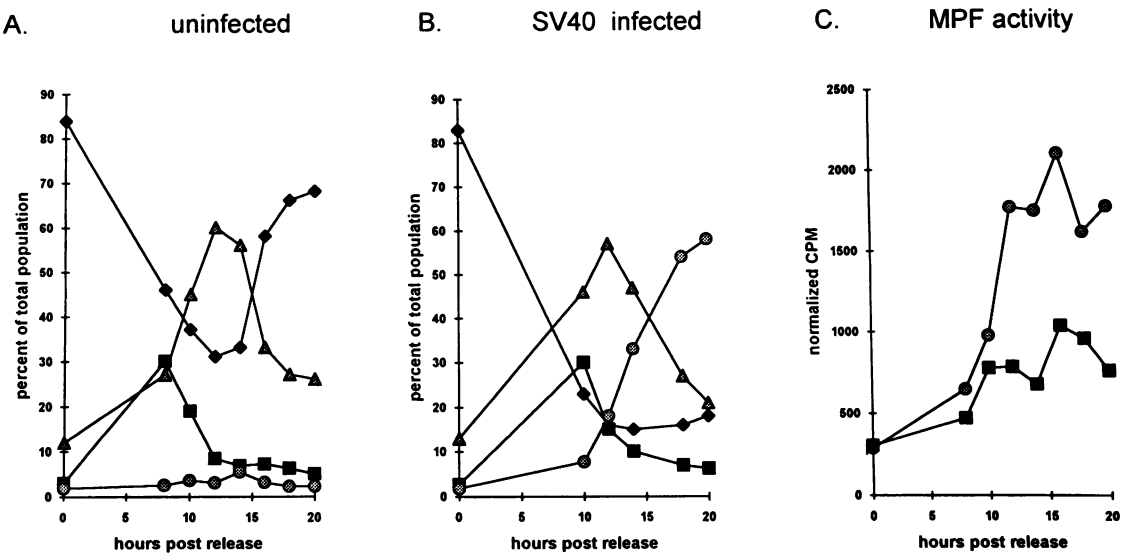

FIG. 2. Cell cycle progression and MPF kinase activity of uninfected and SV40-infected CV-1 cells. Confluent CV-1 cells were trypsinized and replated or SV40 infected prior to mimosine block and release, as described in the legend to Fig. 1. Infected populations were greater than 80% T antigen positive by 10 h postrelease. The cell cycle distributions of uninfected (A) and SV40-infected (B) cultures were determined by FCM analysis. G_1 phase, diamond; S phase, square; G_2 phase, triangle; $>G_2$ phase, circle. (C) Anti-cyclin B-associated H1 kinase activity of cell lysates from uninfected (squares) and infected (circles) cultures. The H1 kinase activity of uninfected confluent cultures was equivalent to that seen at 0 h.

in $>G_2$ by 18 to 20 h postinfection (Fig. 2B). As shown in Fig. 3B, cyclin B-associated $p34^{cdc2}$ is barely detectable in confluent and late-G_1-phase-infected cultures but increases threefold between 8 and 18 h postrelease, as cells move through G_2 and into $>G_2$. The $p34^{cdc2}$ was almost exclusively the more slowly migrating, phosphorylated form at all time points in the infected cultures.

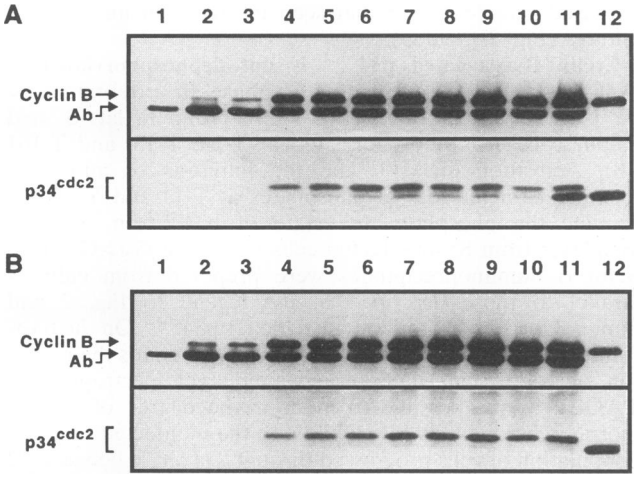

FIG. 3. Cyclin B-associated $p34^{cdc2}$ in uninfected and SV40-infected CV-1 cells. Cell lysates were prepared from uninfected (A) and SV40-infected (B) cultures parallel to those described in the legend to Fig. 2 and immunoprecipitated with anti-human cyclin B1 (Pharmingen). Precipitates were resolved by SDS-PAGE and immunoblotted. The upper half of each filter was reacted with anti-human cyclin B1 and then with alkaline phosphatase-conjugated goat anti-mouse antibody. The lower halves were reacted with anti-$p34^{cdc2}$ G6 antibody and then with alkaline phosphatase-conjugated goat anti-rabbit antibody. Lanes: 1, Anti-cyclin B antibody only; 2, confluent cells; 3 to 10, mimosine-released cultures for 0 h (lanes 3), 8 h (lanes 4), 10 h (lanes 5), 12 h (lanes 6), 14 h (lanes 7), 16 h (lanes 8), 18 h (lanes 9), 20 h (lanes 10), and 20-h release in the presence of colcemid (lanes 11); 12, lysate of mitotic shake of independent uninfected culture reacted with $p13^{suc1}$-Sepharose. Ab, immunoglobulin G1 heavy chain.

To trap mitotic cells and enhance for active MPF complexes, plates of infected and uninfected cells were released from mimosine into colcemid and harvested at 20 h post-mimosine release. In the uninfected cultures, there was an enhanced accumulation of the faster-migrating, underphosphorylated form of cyclin B-associated $p34^{cdc2}$ (Fig. 3A, lane 11). This form of $p34^{cdc2}$ comigrated with active $p34^{cdc2}$ obtained by $p13^{suc1}$-Sepaharose affinity from a mitotic shake of uninfected cells (Fig. 3A, lane 12). In the SV40-infected culture released from mimosine into colcemid, the faster-migrating form of cyclin B-associated $p34^{cdc2}$ was not detected (Fig. 3B, lane 11).

To determine whether the more slowly migrating form of $p34^{cdc2}$ was phosphorylated on tyrosine, an anti-phosphotyrosine antibody was used to probe blots of anti-cyclin B and $p13^{suc1}$-Sepharose-precipitated lysates. Confluent CV-1 cells were infected or stimulated into the cell cycle by trypsinization and harvested at 8 and 16 h post-mimosine release. In both SV40-infected (Fig. 4, lanes 5 to 7) and uninfected (Fig. 4, lanes 2 to 4) cultures, the more slowly migrating form of cyclin B-associated $p34^{cdc2}$ is positive for phosphotyrosine. Tyrosine-phosphorylated $p34^{cdc2}$ is not present in a mitotic population obtained by mitotic shake (Fig. 4, lane 10), even though the underphosphorylated form of $p34^{cdc2}$ is easily detected in the same lysate with G6 antibody (Fig. 4, lane 13). The presence of multiple phosphotyrosine bands in p13-precipitated extracts from SV40-infected (Fig. 4, lane 8) and uninfected colcemid-treated cells (Fig. 4, lane 9) suggests additional tyrosine-phosphorylated, non-cyclin B-associated Cdks.

DISCUSSION

FCM of SV40-infected CV-1 cells demonstrates that following the completion of S phase, infected cells bypass mitosis and initiate a second phase of cellular DNA synthesis. To understand the mechanisms of altered cell cycle control allowing a

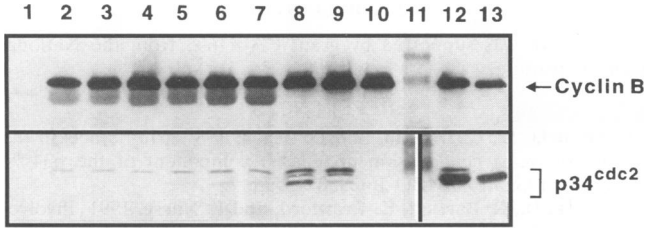

FIG. 4. Tyrosine phosphorylation of cyclin B-associated $p34^{cdc2}$. Synchronized uninfected and SV40-infected cultures were prepared by mimosine release as described in the legend to Fig. 1. Lysates were precipitated with rabbit anti-cyclin B or $p13^{suc1}$-Sepharose, resolved by SDS-PAGE, and immunoblotted. The upper half of the filter was reacted with anti-human cyclin B1 and then with alkaline phosphatase-conjugated goat anti-mouse. The lower half of lanes 1 to 10 was reacted with anti-phosphotyrosine antibody and alkaline phosphatase-conjugated goat anti-mouse. The lower half of lanes 12 and 13 was reacted with anti-$p34^{cdc2}$ (G6) and alkaline phosphatase conjugated goat anti-rabbit. Lane 1, uninfected 16-h release immunoprecipitated with normal rabbit serum; lanes 2 to 7, immunoprecipitation with rabbit anti-cyclin B; lane 2, uninfected 8-h release; lane 3, uninfected 16-h release; lane 4, uninfected 16-h release in colcemid; lane 5, SV40-infected 8-h release; lane 6, infected 16-h release; lane 7, infected at 36 h postinfection without mimosine; lanes 8 to 10 and 12 and 13, precipitation with $p13^{suc1}$-Sepharose; lane 8, SV40-infected 16-h release; lanes 9 and 12, uninfected, colcemid treated; lanes 10 and 13, uninfected, colcemid treated, mitotic shake; lane 11, prestained molecular weight markers.

second S phase within a single cell cycle, it is important to determine how mitosis is prevented and how DNA synthesis is reinitiated. In this report, mechanisms potentially involved in the prevention of mitosis were examined.

Inhibition of appropriate pathways leading to the synthesis or activation of MPF could be blocked through the loss of either the cyclin B or the $p34^{cdc2}$ subunit or by disruption of normal $p34^{cdc2}$ phosphorylation or dephosphorylation. Immunoblot analysis of lysates from SV40-infected CV-1 cells bypassing mitosis demonstrated that the cells contained near-normal levels of cyclin B and $p34^{cdc2}$. On the basis of electrophoretic migration, cyclin B-associated $p34^{cdc2}$ was in the hyperphosphorylated form. In addition, this hyperphosphorylated form of cyclin B-associated $p34^{cdc2}$ from infected cells contained phosphotyrosine, suggesting an inactive, pre-MPF complex. These results support the explanation that SV40 interferes with the normal sequence of events resulting in MPF activation. However, the alteration of events following MPF activation or in other pathways has not been excluded and may also play a role in preventing mitosis.

The H1 kinase activity of anti-cyclin B immunoprecipitates from SV40-infected cells was reduced relative to uninfected mitotic populations but was not absent. Since $p34^{cdc2}$ is currently the only Cdk known to associate with cyclin B in vivo, the data indicate that immunoprecipitated MPF from infected cells is at least partially activated. There are several possible explanations for this observation. First, inactive MPF complexes may have been activated in vitro during the kinase assay, because of the activity of phosphatases. This is supported by the observation that anti-cyclin B immunoprecipitates from SV40-infected cells run directly on SDS-PAGE show no underphosphorylated $p34^{cdc2}$. Dephosphorylation of tyrosine during immunoprecipitation has been previously reported (31). The associated phosphatase activity is likely to be Cdc25, which is known to form a complex with cyclin B-$p34^{cdc2}$ (10). Second, SV40-infected cells may contain subthreshold levels of active MPF. This is not likely, considering that MPF activity is known to self-amplify. Active MPF phosphorylates and thereby activates Cdc25, which dephosphorylates additional cyclin B-associated $p34^{cdc2}$ (15). Partial activation of MPF in infected cells would require that Cdc25 activation be restricted or that increased Cdc25 activity be compensated for by increased phosphorylation of $p34^{cdc2}$ by the protein kinase Wee1. Third, MPF may be expressed, but for a shorter time than in mitotic cells. Normally, increased MPF activity coincides with the initiation of mitosis and the loss of MPF activity corresponds with the metaphase-anaphase transition. It is unlikely that this explanation alone is responsible for decreased MPF activity in infected cells. In the absence of any other alterations, it would be expected that increased MPF activity would result in the initiation of nuclear membrane breakdown and chromosome condensation. However, these morphological changes are not observed in infected cells. Fourth, cyclin B-associated H1 kinase activity may be present in infected cells, but restricted to the cytoplasm. Recent experiments have shown that Wee1, which is present in the nucleus throughout the cell cycle, can prevent mitosis by protecting the nucleus from active cyclin B-$p34^{cdc2}$ complexes in the cytoplasm (14). Finally, the H1 kinase activity associated with cyclin B may not be $p34^{cdc2}$. Although $p34^{cdc2}$ is the only Cdk demonstrated to bind to cyclin B in vivo, mixing experiments in vitro have produced cyclin B-associated Cdk2 with kinase activity (4). This raises the possibility that viral infection may result in the formation of alternative cyclin B-Cdk complexes.

Inactive MPF complexes containing cyclin B and hyperphosphorylated $p34^{cdc2}$ have also been identified in cells delayed in G_2 phase because of DNA damage. In eukaryotes, the presence of DNA damage or unreplicated DNA triggers feedback controls that prevent entry into mitosis. CA46 human lymphoma cells which have been released from a G_1/S block and treated with nitrogen mustard during early S phase are delayed in passage through S and G_2 and have a reduced rate of entry into G_1 phase. Treatment with nitrogen mustard followed by pentoxifylline eliminates the S-phase delay and reduces the delay in G_2 phase. The nitrogen mustard-induced delay in G_2 phase is accompanied by a reduced level of MPF kinase activity, which is restored in cells treated with pentoxifylline (34). Likewise, Chinese hamster ovary (CHO) cells exposed to the topoisomerase inhibitor etoposide arrest in G_2 phase, with reduced levels of $p34^{cdc2}$ kinase activity (28). In G_2-delayed cultures of both cell types, cyclin B-associated $p34^{cdc2}$ remains in the hyperphosphorylated form. Hyperphosphorylated $p34^{cdc2}$ associated with cyclin B is also found in mammalian cells with unreplicated DNA. BHK cells blocked in S phase with hydroxyurea accumulate complexes of cyclin B and hyperphosphorylated $p34^{cdc2}$. Treatment of hydroxyurea-blocked BHK cells with caffeine or okadaic acid results in dephosphorylation of $p34^{cdc2}$ and the induction of premature chromosome condensation (45).

The generation of cells with tetraploid-polyploid DNA contents is found naturally during the polytenization of insect cells. However, in contrast to SV40-infected CV-1 cells, in which an inactive MPF complex is assembled, *Drosophila* embryo cells undergoing polytenization do not express cyclins A or B (26), Cdc2 (17), or Cdc25 (7). In addition, *Drosophila* that are mutant for the *cdc25* homolog, *string*, undergo normal polytenization (42). Broek et al. (2) describe *cdc2* mutants of *Schizosaccharomyces pombe* that block in G_2 phase at nonpermissive temperatures but reenter S phase when returned to a permissive temperature. At nonpermissive temperatures, the G_2-phase form of $p34^{cdc2}$ is degraded and "memory" of G_2

phase is lost. Therefore, in both systems described above, cells that bypass mitosis do not have cyclin B-associated $p34^{cdc2}$. SV40-infected CV-1 cells are distinct in having an inactive MPF complex as they bypass mitosis and proceed through a second S phase.

After the completion of S phase, the acquisition of a tetraploid DNA content is dependent not only on the prevention of mitosis but also on the continuation of DNA synthesis. Two general patterns of continued DNA synthesis can be imagined. First, a gap phase may separate S phase from the initiation of tetraploid S phase. This gap could include G_1 and/or G_2 phase or be a unique G phase. It may be recognizable by the appearance of markers characteristic of G_1 or G_2 phase. Second, there may be a continuous S phase, including diploid and tetraploid S phases without an intervening gap.

Current evidence supports the separation of successive S phases by a functional gap of short duration. First, as can be seen in parallel mimosine-released cultures (Fig. 2), SV40-infected cells enter $>G_2$ at about the same time that uninfected cells leave G_2/M and enter G_1 phase. This indicates that infected cells do not pass through a G_1 phase of standard length prior to entering tetraploid S phase. Second, CV-1 cells infected with the *ts*A30 mutant of SV40 at the nonpermissive temperature accumulate at a G_2 DNA content but do not enter $>G_2$ (8). In contrast, in *ts*A30-infected cultures maintained at the permissive temperature, T-antigen-positive cells behave as a wild type and acquire $>G_2$ DNA content. The G_2 block or delay at nonpermissive temperature indicates that there is a T-antigen-dependent checkpoint at the completion of S phase. Continued DNA synthesis and entry into $>G_2$ is dependent on wild-type T-antigen function. Finally, entry into $>G_2$ is associated with the appearance of hypophosphorylated pRB (9). In the normal cell cycle, hypophosphorylated pRB is restricted to G_1 phase. pRB is phosphorylated late in G_1 phase and dephosphorylated at the metaphase-anaphase transition. The mechanism of pRB dephosphorylation and its role, if any, in entry into a second S phase are not known. However, the dephosphorylation of pRB as infected cells pass into $>G_2$ indicates that the cells are not simply undergoing an extended S phase. Considering that pRB dephosphorylation is ordinarily associated with the metaphase-anaphase transition, the appearance of hypophosphorylated pRB in $>G_2$ cells may represent an uncoupling of cell cycle regulatory mechanisms from the cytological changes of mitosis.

Although it is not known why SV40 induces successive rounds of cellular DNA synthesis, it can be speculated that keeping the cells in a nearly continuous S phase is an efficient means of producing viral progeny. The results presented here indicate that SV40 does not have an entirely unique means of preventing mitosis, since the inactive MPF complexes of SV40-infected cells resemble the MPF complexes of cells delayed in G_2 phase because of DNA damage. It has been suggested that feedback controls maintaining the dependency of mitosis on the completion of S phase do not monitor total DNA content, but rather sense replication machinery (27). If so, the presence of replicating viral DNA in cells that have completed one round of cellular DNA synthesis may trigger feedback controls preventing the activation of MPF. G_2-phase feedback controls may also be stimulated because of the clastogenic action of T antigen (38). Alternatively, MPF activation may be inhibited by interaction of T antigen with components of pathways regulating Wee1 or Cdc25. Future experiments will examine the influence of SV40 infection on the protein kinases and phosphatases that control the phosphorylation state of cyclin B-associated $p34^{cdc2}$.

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