

Influenza A Virus Replication Induces Cell Cycle Arrest in G₀/G₁ Phase[∇]

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Many viruses interact with the host cell division cycle to favor their own growth. In this study, we examined the ability of influenza A virus to manipulate cell cycle progression. Our results show that influenza A virus A/WSN/33 (H1N1) replication results in G₀/G₁-phase accumulation of infected cells and that this accumulation is caused by the prevention of cell cycle entry from G₀/G₁ phase into S phase. Consistent with the G₀/G₁-phase accumulation, the amount of hyperphosphorylated retinoblastoma protein, a necessary active form for cell cycle progression through late G₁ into S phase, decreased after infection with A/WSN/33 (H1N1) virus. In addition, other key molecules in the regulation of the cell cycle, such as p21, cyclin E, and cyclin D1, were also changed and showed a pattern of G₀/G₁-phase cell cycle arrest. It is interesting that increased viral protein expression and progeny virus production in cells synchronized in the G₀/G₁ phase were observed compared to those in either unsynchronized cells or cells synchronized in the G₂/M phase. G₀/G₁-phase cell cycle arrest is likely a common strategy, since the effect was also observed in other strains, such as H3N2, H9N2, PR8 H1N1, and pandemic swine H1N1 viruses. These findings, in all, suggest that influenza A virus may provide favorable conditions for viral protein accumulation and virus production by inducing a G₀/G₁-phase cell cycle arrest in infected cells.

Many viruses facilitate their own replication by interacting with the host cell cycle. Examples can be found among DNA viruses, retroviruses, and RNA viruses. DNA viruses, whose primary site of replication is the nucleus, have been studied most extensively in regard to cell cycle control. Some small DNA viruses lacking their own polymerase, such as simian virus 40 (6, 10), adenovirus (8, 23), and human papillomavirus (54), encode proteins that promote the entrance of cells into S phase in order to support viral genome synthesis. Other large DNA viruses, such as herpesviruses, are able to elicit cell cycle arrest in the G₀/G₁ phase so that competition for cellular DNA replication resources is avoided (12). Similar to the case for DNA viruses, cell cycle regulation has been observed for retroviruses, which also replicate in the nucleus. The Vpr protein

of human immunodeficiency virus type 1 is responsible for inducing cell cycle arrest in G₂/M phase, when the expression of the viral genes is most optimal (19, 21). Increasingly, RNA viruses, whose primary site of replication is normally the cytoplasm, have also been demonstrated to interfere with the host cell cycle. In the coronavirus family, infectious bronchitis virus (IBV) induces a G₂/M-phase arrest in infected cells to favor viral replication (7), while mouse hepatitis virus (MHV) replication and some severe acute respiratory syndrome coronavirus (SARS-CoV) proteins are able to induce cell cycle arrest in G₀/G₁ phase (3, 57–59).

G₁-phase progression is regulated by cyclin-Cdk complexes and the phosphorylation of the downstream retinoblastoma (Rb) protein. The cyclin D-Cdk4/6 complex is responsible for G₁-phase progression, while the cyclin E-Cdk2 complex is required for S-phase entry and DNA replication (35). The Rb protein is firstly hypophosphorylated by the cyclin D1-Cdk4/6 complex and then hyperphosphorylated by the cyclin E-Cdk2 complex, which allows E2F, identified as a potent transcriptional activator required for cell proliferation, to dissociate from the phosphorylated Rb protein and be activated. Thus, genes essential for DNA synthesis can be transcribed, and cells are allowed to progress into the S phase (30, 53). CKI molecules are also involved in G₁-phase progression, as inhibitors of active

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Cdk-cyclin complexes. CKIs can be grouped into two families. The Ink4 family, consisting of p16^{Ink4A}, p15^{Ink4B}, p18^{Ink4C}, and p19^{Ink4D}, especially targets Cdk4/Cdk6. The other family, the Cip/Kip family, composed of p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, has a wide spectrum of inhibitory effects on G₁ Cdk-cyclin complexes (33).

Among the studies on RNA viruses and cell cycle interaction, some positive-strand RNA viruses, namely, the coronavirus family, have been investigated extensively. Little is known about other RNA viruses, especially negative-strand RNA viruses. As a very important zoonotic negative-strand RNA virus, influenza virus has caused global concern because of its antigenic variability and pandemic potential (2). With the outbreaks of highly pathogenic avian virus and swine-origin influenza A virus H1N1 (S-OIV H1N1) in the human population, intensive research on influenza virus is under way and is urgently needed (5, 16, 45). Previous studies have demonstrated that influenza virus is a cytolytic virus that induces apoptosis in many cell types (11, 22, 48). Some viral proteins and many cell signaling molecules have been found to be involved in induction of cell death during influenza virus infection (24, 29, 31). A common phenomenon of p53 activation in influenza virus-induced cell death has been observed in different cell types (39, 49, 50, 60). However, p53, as a multifunctional transcription factor, not only is essential in the apoptosis process but also plays a role in cell cycle regulation (44). The binding of activated p53 to DNA results in expression of p21 (31), and subsequent binding of p21 to the cyclin E-Cdk2 complex inhibits the complex's activity and blocks the G₁/S transition (40). Cross talk between apoptosis and the cell cycle has been proposed because they share a number of regulatory molecules in their mechanisms (14). It is therefore reasonable to ask whether influenza virus infection affects the cell cycle process in a manner other than apoptosis induction.

In this study, we examined potential effects on the cell cycle during influenza virus infection. The data show that influenza A virus A/WSN/33(H1N1) replication induces cell cycle arrest in G₀/G₁ phase. Furthermore, we are the first to suggest that arresting cells in G₀/G₁ phase produces favorable conditions for viral protein expression and for production of influenza A virus. Additional analyses of several different strains and subtypes indicated that cell cycle regulation ability may be a common strategy exploited by influenza A viruses.

MATERIALS AND METHODS

Viruses and cells. A/California/07/09 (H1N1), A/Guangzhou/333/99 (H9N2), and A/Donghu/312/2006 (H3N2) viruses, kindly provided by the Chinese Center for Disease Control and Prevention, were propagated in 10-day-old specific-pathogen-free (SPF) chicken embryos. A/WSN/33 (H1N1) virus was rescued by plasmid-based reverse genetics, followed by two rounds of plaque purification and propagation on Madin-Darby canine kidney (MDCK) cells for stock development (41). A/PR/8/34 (H1N1) virus was generated by plasmid-based reverse genetics and was kindly provided by Robert G. Webster (St. Jude Children's Research Hospital, University of Tennessee). This was followed by plaque purification twice on MDCK cells and propagation in SPF chicken embryos. Viral RNAs from all strains of viruses were extracted by using a QIAamp viral RNA kit (Qiagen), followed by reverse transcription-PCR. Viral cDNAs were further subjected to commercial sequencing. Virus titers determined for infection were calculated by plaque assay titration on A549 cells. The inactivation of the viruses was achieved using UV light irradiation (0.36 J). MDCK cells and human lung adenocarcinoma epithelial A549 cells were purchased from ATCC and were

maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone).

Synchronization of cells. Subconfluent cultures of A549 cells were synchronized in the G₀ phase by serum deprivation. Approximately 5 × 10⁵ cells were plated in a 6-well plate and maintained in medium containing no serum for 48 h. For mitotic arrest, A549 cells were treated with nocodazole (M1404; Sigma) at a final concentration of 400 ng/ml.

Infection. For cell cycle analysis, cells were mock infected or infected with influenza virus at a multiplicity of infection (MOI) of 2. After 1 h of virus adsorption, cells were treated with medium containing 10% FBS and harvested at various times postinfection (p.i.) for cell cycle and Western blot analyses. For comparison of viral protein expression and progeny virus production in different cell cycle phases, cells were infected with the virus at an MOI of 0.1. After 1 h of virus adsorption, the medium was restored to maintain cells in different phases. Ten hours after infection, the cells were harvested, and nucleoprotein (NP) and NS1 protein expression was detected by Western blot analysis. The viruses in the supernatant were titrated by plaque assay on MDCK cells.

Immunofluorescence staining. A total of 10⁶ cells were collected after infection and fixed with 4% paraformaldehyde (PFA) followed by permeabilization with 0.3% Triton X-100. After permeabilization, influenza A virus NP was first stained using an anti-NP polyclonal antibody. Goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) was used as a secondary antibody. The cells were then analyzed by flow cytometry.

Cell cycle analysis by flow cytometry. Nuclear DNA content was measured using propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis as described previously (3). Briefly, adherent A549 cells were collected by treatment with trypsin and were then washed with phosphate-buffered saline (PBS). The cells were fixed in 1 ml of cold 70% ethanol overnight at 4°C and resuspended in staining buffer (50 μg/ml PI [Sigma], 20 μg/ml RNase in PBS) for 20 min at room temperature. PI-stained cells were then analyzed using FACS (FACScan; BD), and at least 30,000 cells were counted for each sample. Data analysis was performed by using ModFit LT, version 2.0 (Verity Software House).

Western blot analysis. Virus-infected and mock-infected cells were collected at various times after influenza A virus infection and were washed once with PBS. Cells were lysed directly in sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue), followed by being boiled for 10 min. Whole-cell lysates were further subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Bio-Rad) and detected with corresponding primary and secondary antibodies. The exposure was developed with an enhanced chemiluminescence kit (Pierce).

Antibodies. The following mouse monoclonal antibodies were used in Western blot analyses: anti-phospho-Rb(Ser795) (Sigma), anti-cyclin D1 (Santa Cruz), anti-cyclin E (Thermo), and anti-p21 (Santa Cruz). Actin was detected with rabbit anti-actin polyclonal antibody (Sigma). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was purchased from Sigma, and HRP-conjugated anti-rabbit IgG was purchased from Southern Biotech. FITC-conjugated anti-rabbit IgG was purchased from Molecular Probes. Anti-NP polyclonal antibody and a polyclonal antibody against nonstructural protein 1 (NS1) were purchased from Antibody Research Centre, Shanghai Institutes for Biological Sciences.

Statistical and densitometric analyses. Statistics were analyzed using Student's *t* test. Data are presented as means and standard deviations (SD). *P* values of <0.05 were considered statistically significant. The densities of bands on Western blots were analyzed by using Image J software (NIH).

RESULTS

More cells accumulate in G₀/G₁ phase in A/WSN/33 (H1N1) influenza A virus-infected cells. Based on the observation that influenza virus causes apoptosis and increases p53 and p21 expression levels (29), we asked whether influenza viruses can regulate the cell cycle, especially at the G₁/S transition. To address this question, A549 cells were infected with A/WSN/33 (H1N1) at an MOI of 2, avoiding the quick occurrence of cytopathic effect but showing a high infectivity (~95% at 24 h postinfection) (Fig. 1A). Cells were collected at 24 h postinfection, and the cell cycle was analyzed by flow cytometry. As suspected, we were able to observe an obvious accumulation of cells in G₀/G₁ phase in infected cells (Fig. 1B). The quantita-

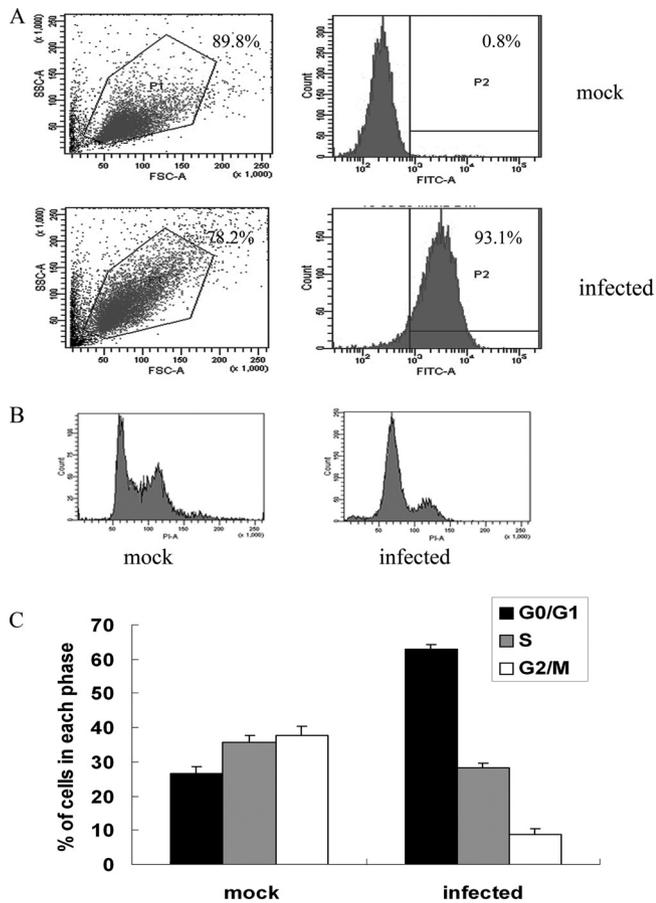


FIG. 1. G_0/G_1 -phase accumulation induced by influenza A virus A/WSN/33 (H1N1) infection. A549 cells were mock infected (mock) or infected with A/WSN/33 (H1N1) virus (infected) at an MOI of 2. At 24 h postinfection, cells were collected and analyzed by flow cytometry. (A) The mock-infected cells and virus-infected cells were stained with anti-NP antibodies, and thus the infection efficiency was evaluated by the percentage of NP-positive cells (P2) compared to gated cells (P1). (B) The mock-infected cells and virus-infected cells were stained with PI, and the cell cycle profiles at 24 h postinfection are shown. (C) The histograms were analyzed by the ModFit LT program to determine the percentage of cells in each phase of the cell cycle. The results are presented as means (+SD) for three independent experiments.

tive analysis revealed a 30% increase of the cell population in G_0/G_1 phase in virus-infected cells compared to that in mock-infected cells (Fig. 1C). These data suggest that the A/WSN/33 (H1N1) virus is able to induce a G_0/G_1 -phase accumulation in infected cells.

Entry of influenza virus-infected cells into S phase is prevented. To understand more of this accumulation, synchronized cells were monitored for cell cycle progress after infection. Synchronized cells were obtained by serum starvation for 48 h and then were infected with the virus for 1 h. Serum mitogenic stimulation with 10% FBS was performed following the infection to trigger cell cycle reentry. Twenty-four to 28 h after the serum was added, mock-infected A549 cells progressed gradually into the normal cell cycle, as an obvious decrease in G_0/G_1 -phase cells and an increase in S-phase cells were detected. In contrast, the majority of A/WSN/33-infected A549 cells stayed in G_0/G_1 phase during the entire time course after

serum stimulation (Fig. 2A and B). This indicates that virus infection induces G_0/G_1 -phase accumulation by preventing cell cycle entry into the S phase.

To observe this prevention in more detail, nocodazole (which is known to block cell cycle progression in G_2/M phase through disruption of mitotic spindles) was applied to induce G_2/M -phase arrest. A549 cells were treated with nocodazole at 8 h postinfection, when the expression of the viral proteins had already occurred. Cells were harvested and analyzed by flow cytometry after another 16 h. The data showed that mock-infected cells progressed normally through G_0/G_1 to G_2/M phase but stopped at G_2/M phase because of mitotic block. Only 4.6% of mock-infected cells remained in the G_0/G_1 phase after 16 h of nocodazole treatment. However, among the A/WSN/33-infected cells, 50% stayed in G_0/G_1 phase, showing little movement into S phase (Fig. 3A and B). The results suggest that nocodazole is a useful chemical for measuring G_0/G_1 -phase arrest. For this reason, nocodazole treatment was applied to study G_0/G_1 arrest in all later experiments.

G_0/G_1 -phase arrest of the cell cycle depends upon the replication of influenza virus. To examine whether cell cycle arrest by influenza virus is dependent on virus replication, the virus was irradiated with 0.36 J of UV light, which will fully retain hemagglutinin (HA) activity (determined by hemagglutination assay [data not shown]) and totally destroy infectivity (25). The cells were mock infected or infected with intact virus or UV-treated virus for 8 h and then were treated with nocodazole as described above. The results showed that a significant G_0/G_1 arrest was detectable only in cells infected with intact virus, not in mock-infected cells or cells infected with UV-treated virus (Fig. 4A). The UV inactivation of virus was further confirmed by the absence of NP expression, while in cells infected with intact virus, NP was expressed normally (Fig. 4B). Thus, we concluded that the G_0/G_1 cell cycle arrest resulted from replication of influenza virus but not from binding to the cellular receptors.

Key molecules regulating the cell cycle in influenza A virus-infected cells. There are many host molecules involved in regulation of the G_1/S transition, such as cyclin-Cdk complexes, pRb, and CKI molecules (42). In order to find the signaling pathway and key molecules responsible for influenza virus-induced cell cycle arrest, we examined the expression profiles of host G_1/S transition proteins. A549 cells were infected with influenza A virus A/WSN/33 (H1N1) and then assayed for expression of these cellular proteins by Western blotting at 8 h, 12 h, and 24 h postinfection, using corresponding antibodies. Among the molecules investigated, there was a significant increase of p21 and decreases of cyclin E and cyclin D1 in virus-infected cells compared to mock-infected cells (Fig. 5). The phosphorylation status of Rb, a downstream regulatory protein of the cyclin-Cdk complex, was also examined. The results in Fig. 5 show that there was a great decrease in the amount of hyperphosphorylated Rb after infection. The protein expression pattern indicates that the amounts of the proteins responsible for cell cycle progression from G_0/G_1 to S phase decrease significantly in virus-infected cells.

G_0/G_1 -phase-synchronized cells facilitate viral protein expression and progeny virus production. The data above indicated a cell cycle arrest in G_0/G_1 phase induced by influenza virus replication. However, it was not clear whether this strat-

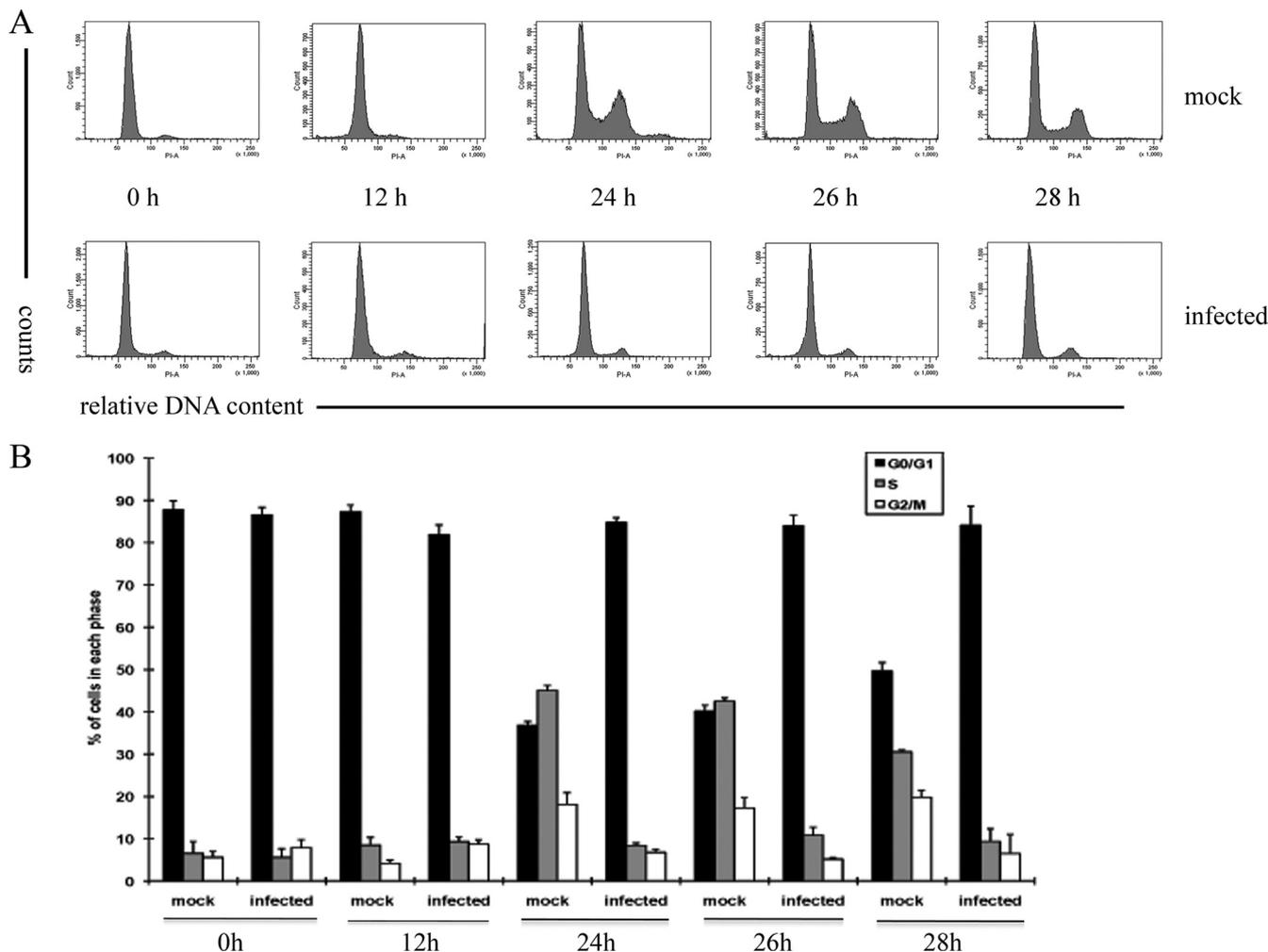


FIG. 2. Influenza A virus A/WSN/33 infection prevents cell reentry into S phase. (A) Serum-starved A549 cells were mock infected (mock) or infected with A/WSN/33 (infected) at an MOI of 2. After 1 h of virus adsorption, medium containing 10% FBS was added to the cells, and cell cycle profiles at the indicated times p.i. were determined by FACS analysis. The data are from one of three independent experiments. (B) The histograms were analyzed by the ModFit LT program, and the percentage of cells in each phase of the cell cycle is shown. The results are presented as means (+SD) for three experiments.

egy executed by the virus is beneficial for the virus. We therefore compared the accumulation levels of viral NP and NS1 in infected cells among G₀/G₁-phase-synchronized cells and, as controls, normal unsynchronized cells and G₂/M-phase-synchronized cells. For G₀/G₁-phase synchronization, cells were cultured in medium containing no serum for 48 h and then infected with A/WSN/33 virus. For G₂/M-phase synchronization, the same number of cells was treated with nocodazole for 16 h and then infected. As shown in Fig. 6A, 82.7% of the cells were successfully blocked in G₀/G₁ phase among G₀/G₁-phase-synchronized cells, while only 40.1% of the cells were in G₀/G₁ phase among normal unsynchronized cells. As for G₂/M synchronization, 88.9% of the cells were in G₂/M phase, with <10% of the cells in G₀/G₁ phase. Cells were then infected with A/WSN/33 virus at the same time, at a low MOI of 0.1. An additional 10 h later, infected cell lysates and supernatants from these three groups were harvested individually. NP and NS1 expression in each group of cells was evaluated by Western blot analysis. The expression levels of both viral proteins in

G₀/G₁-phase-synchronized cells were significantly higher than those in G₂/M-phase-synchronized cells. As a control to eliminate any possible influence of nocodazole treatment, unsynchronized cells with a medium-sized population of G₀/G₁-phase cells showed medium expression levels of viral proteins compared to G₀/G₁- and G₂/M-phase-synchronized cells (Fig. 6B). In order to determine whether the increase in viral protein expression would result in facilitation of progeny virus production, supernatants from each group of cells were examined for progeny virus titers. As expected, more progeny virus production (~7.40 × 10⁴ PFU/ml) was observed in G₀/G₁-phase-synchronized cells than in unsynchronized cells (~4.90 × 10⁴ PFU/ml) and G₂/M-phase-synchronized cells (~1.12 × 10⁴ PFU/ml), in accordance with the pattern of viral protein expression levels (Fig. 6C). Thus, it is likely that it is easier for influenza virus to express viral protein and produce progeny virus in the G₀/G₁ phase of the cell cycle.

G₀/G₁ arrest is observed for influenza A viruses of different strains. Previous studies reported that different influenza vi-

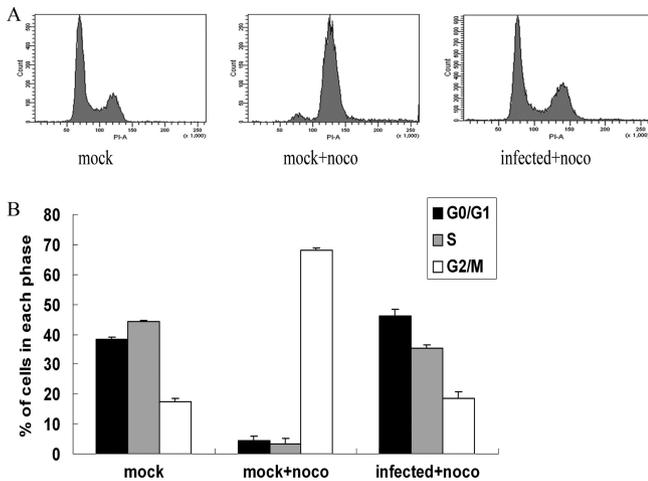


FIG. 3. Infected cells cannot be synchronized to G₂/M phase under nocodazole treatment. (A) A549 cells were mock infected (mock) or infected with A/WSN/33 at an MOI of 2 (infected). At 8 h postinfection, cells were treated with nocodazole (noco) at 400 ng/ml, and cell cycle profiles were determined by FACS analysis at 16 h posttreatment. The data are from one of three independent experiments. (B) The histograms in panel A were analyzed by the ModFit LT program to determine the percentage of cells in each phase of the cell cycle. The results are presented as means (+SD) for three experiments.

ruses may have different functions due to their antigenic variation. We therefore asked whether the cell cycle arrest function is strain specific. Cells were infected with A/WSN/33, A/PR/8/34, and pandemic S-OIV A/California/07/09 (H1N1) viruses, which belong to subtype H1N1 but are of different strains; an avian-origin H9N2 (A/Guangzhou/333/99) virus and an H3N2 (A/Donghu/312/2006) virus (viruses from different subtypes) were also included in the experimental setup. No-

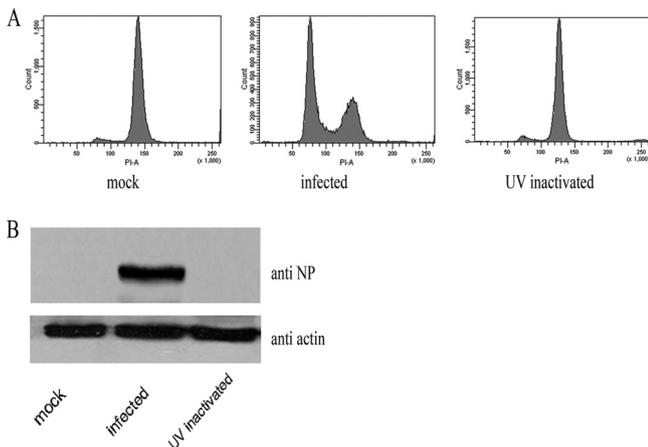


FIG. 4. G₀/G₁-phase arrest of the cell cycle requires replication of the virus. (A) A549 cells were mock infected (mock) or infected with A/WSN/33 virus (infected) or UV-inactivated A/WSN/33 virus (UV inactivated) at an MOI of 2. At 8 h postinfection, the cells were treated with nocodazole, and cell cycle profiles were tested by FACS analysis at 16 h posttreatment. The data are from one of three independent experiments. (B) To ensure that UV-inactivated virus was unable to replicate, total viral NPs from mock-infected, A/WSN/33 virus-infected, and UV-inactivated virus-infected cells were examined by Western blotting. Actin is shown as a loading control.

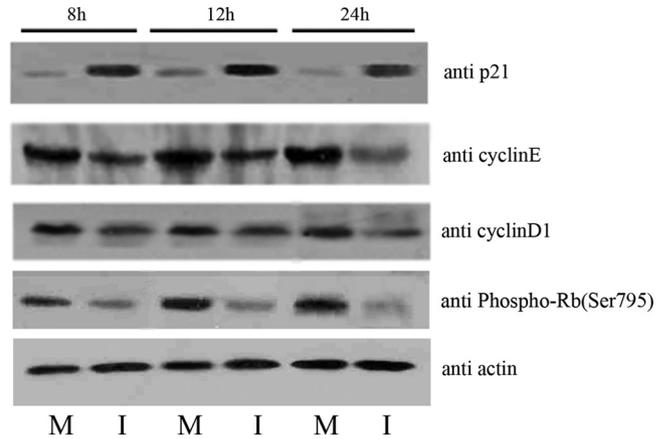


FIG. 5. Western blot analysis of total cellular cell cycle regulatory proteins. A549 cells were mock infected (M) or infected with A/WSN/33 virus at an MOI of 2 (I) and then collected at the indicated times. Cellular proteins were detected by Western blot analysis, and protein amounts were standardized as shown with actin. Several different proteins involved in G₀/G₁-to-S-phase transition control were analyzed, including cyclin D1, cyclin E, regulatory factor p21, and phosphorylated Rb (Ser795), using corresponding antibodies. Actin is shown as a loading control. The experiment was repeated three times, and representative blots are shown.

codazole treatment was applied to the cells according to the protocol used in previous experiments. Cells were collected and analyzed by flow cytometry at 24 h postinfection, when infectivities among strains were similar (data not shown). We found that cells infected with any of the different virus strains showed G₀/G₁-phase accumulation compared to mock-infected cells, although the accumulation extents were a little different. Of the three H1N1 subtype viruses, the A/WSN/33 virus showed the strongest G₀/G₁-phase arrest, while the S-OIV strain showed the weakest arrest. A seasonal H3N2 virus showed a high level of G₀/G₁ accumulation, while the low-pathogenicity avian-origin human H9N2 virus showed a relatively small accumulation effect in human cells (Fig. 7A and B). The data indicate that it may be a common strategy for influenza A virus to arrest the host cell cycle in G₀/G₁ phase.

DISCUSSION

In this study, we investigated whether influenza A virus replication has an effect on cell cycle progress. The results show that influenza A virus replication induces cell cycle arrest in G₀/G₁ phase. Infected quiescent cells failed to reenter the S phase after serum recovery, while the uninfected cells progressed normally in their replication cycle. In addition, uninfected cells progressed and stopped at G₂/M phase after nocodazole treatment, while infected cells were still arrested in G₀/G₁ phase. The expression pattern of some key molecules in regulating the cell cycle was also consistent with the G₀/G₁ arrest profile (40). Further analysis illustrated that G₀/G₁ phase is a favorable condition for viral protein expression and progeny virus production. Moreover, arresting cells in G₀/G₁ phase seems to be a common strategy applied by influenza A viruses of different strains and subtypes.

According to previous reports (60), influenza virus can cause an increase in p53 expression. As a Cdk inhibitor, p21 is under

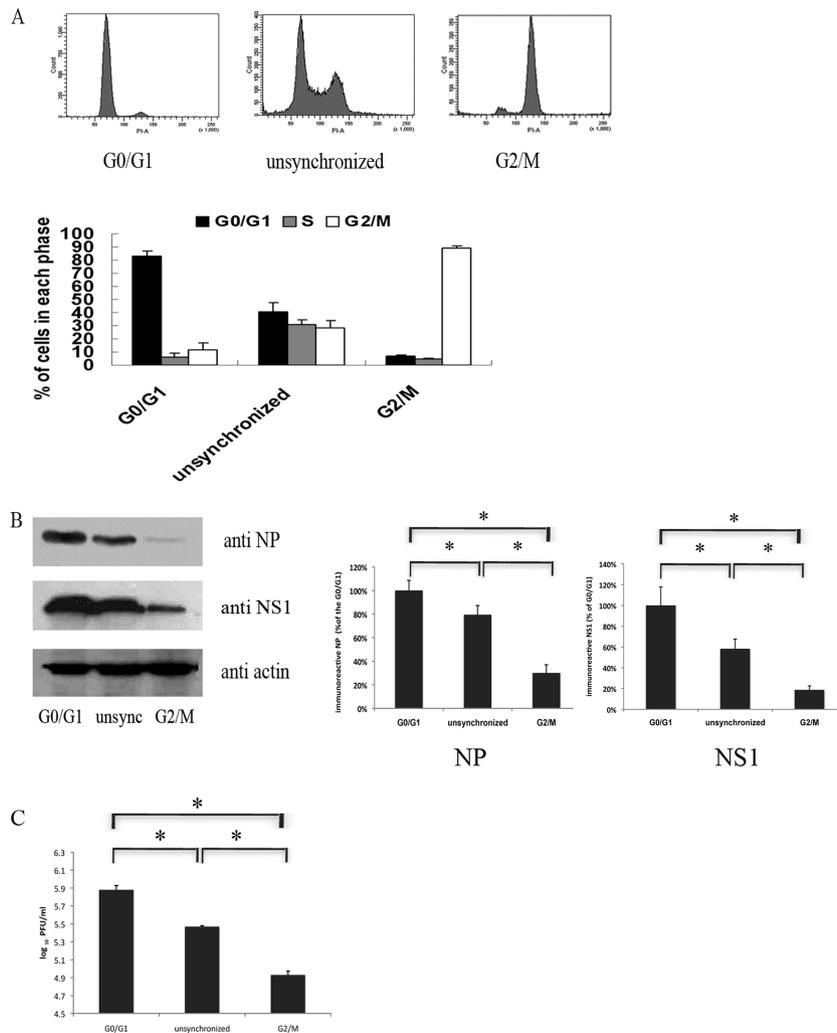


FIG. 6. G₀/G₁-phase-synchronized cells promote viral protein accumulation. Synchronized A549 cells were treated by serum starvation for 48 h or treated with nocodazole for 16 h. The synchronized and unsynchronized cells (unsync) were then infected with A/WSN/33 virus at an MOI of 0.1 for 1 h. After infection, the medium was restored to maintain the respective cell cycle synchronization status. An additional 10 h later, cells were harvested and analyzed. (A) Cell cycle profiles before infection were determined by FACS analysis. The histograms in the upper panel were analyzed by the ModFit LT program to determine the percentage of cells in each phase of the cell cycle. The results are presented as means (+SD) for three experiments. (B) The expression of NP and NS1 was determined by Western blot analysis with anti-NP and anti-NS1 polyclonal antibodies. Actin is shown as a loading control. The data are from one of three independent experiments. The expression of NP and NS1 was quantified with Image J software, and the results are shown in the right panel. The results are presented as means (+SD) for three experiments. *, a statistically significant difference ($P < 0.05$) was observed when the indicated columns were compared. (C) The progeny viruses in the supernatant were titrated by plaque assay on MDCK cells, and quantitative analysis data (PFU/ml) are shown. The results are presented as means (+SD) for three experiments. *, a statistically significant difference ($P < 0.05$) was observed when the indicated columns were compared.

the control of p53 and negatively suppresses the formation of the cyclin E-Cdk2 complex, which is important for the G₁/S transition. Thus, it is possible that influenza virus replication causes G₀/G₁ arrest by increasing p53 expression and then influencing the downstream pathways, including p21 expression. It is also possible that arrest occurs through directly increasing the expression of p21 or preventing proteasome-mediated p21 degradation independent of p53 regulation. Since the expression of both cyclin E and cyclin D1 decreased in our experiments, other mechanisms beyond the p21 pathway that are related to regulation of these two molecules may contribute to arrest at the same time. Similarly, influenza virus infection could inhibit cyclin mRNA transcription or stability.

Influenza virus replication might also inhibit cyclin translation, as host protein synthesis was reported to be suppressed in infected cells (13). A decrease of cyclin E will lead to reduced cyclin E-Cdk2 activity, while a decrease of cyclin D1 will lead to reduced cyclin D-Cdk4/6 activity. The reduced activities of the cyclin E-Cdk2 complex and the cyclin D1-Cdk4/Cdk6 complex will then result in reduced amounts of phosphorylated Rb protein. Therefore, the amount of active E2F accumulated by pRb will decrease, leading to less expression of E2F-regulated genes essential for cell cycle transition from G₀/G₁ phase to S phase (30, 53).

The biological significance of influenza virus-induced cell cycle arrest was supported by more efficient viral protein ex-

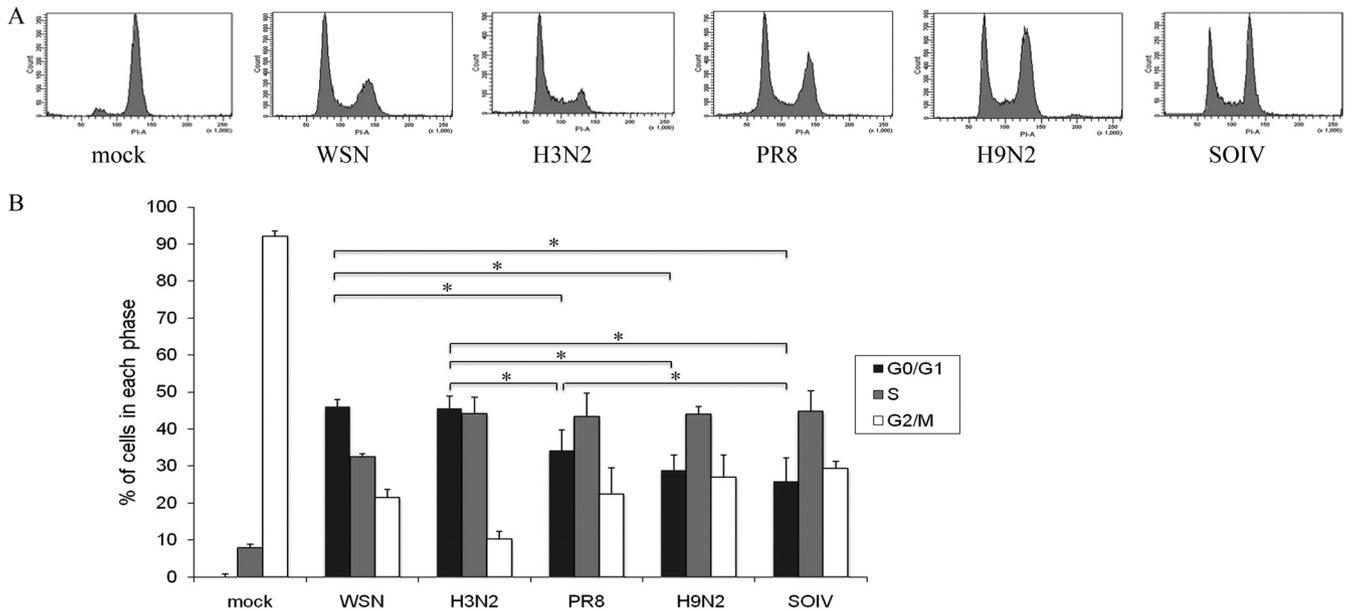


FIG. 7. Cell cycle regulation effects in different strains. (A) Subconfluent A549 cells were mock infected or infected with A/WSN/33 (H1N1), A/PR/8/34 (H1N1), A/California/07/09 (H1N1), A/Guangzhou/333/99 (H9N2), or A/Donghu/312/2006 (H3N2) virus at an MOI of 2 and then treated with nocodazole. Cells were harvested 16 h after nocodazole was added. The cells were then fixed, stained with PI, and submitted to FACS analysis. The data are from one of three independent experiments. (B) The histograms were analyzed with the ModFit LT program to determine the percentage of cells in each phase of the cell cycle. The results are presented as means (+SD) for three experiments. *, a statistically significant difference ($P < 0.05$) was observed when the indicated columns were compared.

pression and progeny virus production, as illustrated in Fig. 6. This benefit for the virus may be explained by several hypotheses, such as increasing the efficiencies of transcription, translation, and virus assembly. First, since influenza A virus transcription requires 5'-capped RNAs as primers derived from cellular transcripts which are transcribed by DNA-dependent RNA polymerase II (Pol II), the activity of Pol II is tightly associated with the efficiency of virus replication (9). It was reported that the transcription activity of Pol II is much higher in the G_0/G_1 phase than in the S and G_2/M phases (56). Thus, influenza virus may arrest the cell cycle to increase the transcription activity of Pol II and therefore favor the transcription of the viral genome (56). Second, the translation of influenza viral proteins is known to be related to host cap-dependent translation activity (17, 36). Since cap-dependent translation is optimal in the G_0/G_1 phase and then decreases gradually because of the impaired function of cap binding protein in mitosis (37), arresting cells in the G_0/G_1 phase will prevent mitosis progress, which should be beneficial for the cap-dependent translation of influenza viral proteins. As a third point, it was proposed by Lin and Lamb (27) that enveloped RNA viruses may delay the cell cycle to favor their assembly because the endoplasmic reticulum (ER) and Golgi apparatus disassemble into vesicles and larger membrane structures break into clusters or remnants during mitosis (28, 51). The ER and Golgi structures are also essential for influenza virus assembly because influenza virus membrane proteins such as HA, neuraminidase (NA), and M2 need to be translated and inserted into the ER and then transported to the cell surface by the *trans*-Golgi network (TGN) (46, 47). Fourth, it is possible that cell cycle arrest in influenza virus infection prevents early death of infected cells. Accumulated data imply links between

cell cycle and apoptosis signaling. A delay of apoptosis always occurs following cell cycle arrest (20, 43). G_0/G_1 cell cycle arrest by influenza virus may be undertaken to avoid early apoptosis of infected cells and therefore gain sufficient time and resources for the viral life cycle. This hypothesis is also consistent with studies which suggest that influenza virus initiates antiapoptotic signaling at early and middle phases of infection to protect cells from fast apoptotic death (60). Finally, G_0/G_1 arrest may also have functional consequences when influenza viruses naturally infect a host organism. It has been reported that noncycling cells are refractory to killing by cytolytic T cells (34). Thus, influenza virus may use a cell cycle arrest strategy to avoid being killed by cytolytic T cells. Moreover, it is also possible that the G_0/G_1 arrest induced by influenza A virus could inhibit the proliferation of infected lymphocytes, as observed for other viruses (55). Overall, our data show that influenza A virus induces a G_0/G_1 -phase arrest which is beneficial for viral protein expression and progeny virus production. The exact mechanism needs to be analyzed further.

Influenza viruses are divided into different subtypes according to their surface glycoproteins. Currently, 16 subtypes of HAs and 9 subtypes of NAs have been identified in nature, and different combinations of certain HAs and NAs result in the existence of many influenza A virus subtypes (15, 52). Even within the same subtype, viruses may show differences among strains. The newly emerging swine-origin influenza virus is an H1N1 subtype, but its antigenic properties are far from those of the regular seasonal H1N1 virus (18). As a result, different subtypes and strains may exhibit similar functions in cell cycle regulation, but their extents of regulation may be different. The strains that we examined in this study, including PR8/H1N1,

WSN/H1N1, S-OIV H1N1, H3N2, and H9N2 viruses, were all able to cause infected cells to accumulate in G₀/G₁ phase, although the arrest levels were not exactly the same. Whether the cell cycle arrest ability is related to the individual pathogenicity of each virus will be interesting to study further. However, our data suggest that cell cycle arrest may be a common strategy to favor virus production, since all influenza viruses share similar transcription, translation, and replication mechanisms.

The exact viral protein that is responsible for the observed cell cycle arrest in G₀/G₁ phase is still unclear. Since there appears to be a linkage between apoptosis and cell cycle control mechanisms (1), we speculate that proteins involved in influenza A virus-induced apoptosis may also take part in the cell cycle arrest. Several influenza virus proteins have already been described to activate intrinsic or extrinsic apoptotic induction pathways (29). NS1, NA, and a rather newly described gene product, PB1-F2, have all been proven to be related to apoptosis (4, 26, 32, 38). Since the strength of cell cycle arrest varies among the strains examined, it is possible that one or more viral proteins of certain strains are involved.

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