The Cell Cycle and Virus Infection

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Summary

A number of different viruses interact with the cell cycle in order to subvert host-cell function and increase the efficiency of virus replication; examples can be found from DNA, retro, and RNA viruses. The majority of studies have been conducted on DNA and retroviruses whose primary site of replication is the nucleus, but increasingly a number of researchers are demonstrating that RNA viruses, whose primary site of replication is normally the cytoplasm, also interfere with the cell cycle. Viral interference with the cell cycle can have a myriad of different effects to improve virus infection, for example to promote replication of viral DNA genomes, or to delay the cell cycle to allow sufficient time for RNA virus assembly. Although cell cycle control is fairly well characterized in terms of checkpoints and control molecules (e.g., cyclins), in recent years several researchers have demonstrated that the nucleolus is also involved in cell cycle control. The nucleolus and associated subnuclear structures can sequester cell cycle regulatory complexes, and nucleolar proteins also have a direct and indirect effect on the cycling cell. Viruses also interact with the nucleolus. In order to study the interactions between a virus and the cell cycle and *vice versa* we have developed and adapted a number of different approaches and strategies. These include determinations of virus yield and measurements of virus replication to flow cytometry and confocal analysis of the host cell. Increasingly we have found that proteomic approaches allow the rapid analysis of a whole plethora of cell cycle proteins that may be affected by virus infection.

Key Words

Cell cycle; virus; infection; confocal microscopy; nucleolus; 2D gel; proteomics.

1. Introduction

A common strategy of viruses is the creation inside the cell of an environment favorable for efficient replication of their genomes; one way of achieving this is by altering the cell cycle. The function of the cell cycle itself comprises a highly con-

served and sequential series of checkpoints and phases ensuring that conditions are suitable for the proper function of that cell and for DNA replication and cytokinesis. There are three categories of viruses, depending on their genome and replication strategy: DNA, retro, and RNA viruses. DNA viruses employ a number of mechanisms to modify and interfere with the cell cycle regulatory machinery. In some cases viruses are adapted to multiply in resting cells, whereas in others they induce proliferation of arrested cells or just wait until the infected cell replicates.

Two main strategies for DNA viruses interfering with host cell cycle control can be distinguished. One has generally evolved in viruses with large genomes with the potential to encode many proteins, including those required for viral DNA replication. A typical example of this strategy is found in the herpesviruses to block cell cycle progression, preventing entry into S-phase (1). The other strategy to impinge on cell cycle control is more typical of DNA viruses with small genomes. Here, virus-encoded proteins, which are not homologs of any known cellular protein, interfere directly with the normal function of cell cycle regulatory components to subvert their activity. Typical examples are the viral oncoproteins that sequester the retinoblastoma (RB) tumor suppressor protein as a first step in inducing S-phase entry by activating the expression of E2F-regulated genes; for example, adenoviruses (2). Disruption of the cell cycle to favor virus replication is not confined to DNA viruses. Retroviruses also disrupt the cell cycle.

Cells infected with human immunodeficiency virus (HIV) do not proliferate but accumulate in the G₂-phase of the cell cycle, resulting in an increase in virus production (3). The viral protein responsible for this has been identified as Vpr (4). Interestingly, Henklein et al. (5) demonstrated that extracellular added Vpr induced G₂-phase arrest, and the authors suggested that free Vpr in the serum of HIV-infected patients may preprogram cells in order to facilitate replication of HIV in infected individuals. Consistent with the perturbation of the cell cycle observed in tissue culture-infected cells, lymphocytes from HIV-infected individuals show high levels of cyclin B and also nucleolar proteins (whose expression is linked to the cell cycle) (6).

Altering the host cell cycle by RNA viruses has not been described extensively in the literature, and the mechanisms of action are generally not well characterized. For the negative-strand RNA viruses, there are several examples of cell cycle control. For instance, measles virus infection results in a G₀ block (7), and the paramyxovirus simian virus V protein prolongs the cell cycle by delaying cells in G₁ and G₂ (8). In the case of positive-strand RNA viruses, the avian coronavirus infectious bronchitis virus (IBV) delays cell growth by inhibiting cytokinesis and also allows cells to accumulate in S/G₂ (9,10). Several examples of different picornaviruses interacting with the cell cycle have been described. More recently Feuer et al. (11) have shown that cells arrested in G₁ or G₁/S produced higher levels of infectious coxsackievirus and viral polyproteins compared with cells in the G₀ phase, or cells blocked at the G₂/M boundary. Feuer et al. (11) suggested that persistence of coxsackie B3 virus (CVB3) in vivo might be dependent on infection of G₀ cells, which do not support replication. If such cells were to reenter the cell cycle, then the virus may reactivate and trigger chronic viral or immune-mediated pathology in the host. Such findings suggest that locally

delivered cell cycle inhibitors could form part of an antiviral therapy, similar to the example of interleukin-2 used to correct cell cycle aberrations in HIV-infected individuals (12).

Viruses also target subnuclear structures involved in cell cycle regulation as part of a strategy to subvert host cell functions such as the cell cycle. For example, many DNA, retro, and RNA viruses target the nucleolus (13,14). The nucleolus and associated proteins are also implicated in (and regulated by) the cell cycle (15). Cajal bodies associated with nucleoli can sequester cyclin-dependent kinase 2 (CDK2) and cyclin E in a cell cycle-dependent manner (16). The concentrations of many nucleolar proteins such as nucleolin (17) are dependent on the cell cycle (18,19). Nucleolin itself can also act as a cell cycle regulator (20).

When studying viruses and the cell cycle, one must consider the interrelationship between the two. The cell cycle will have an effect on virus replication, and virus replication will concomitantly affect the cell cycle. Below we describe experiments that can be used to investigate whether a particular virus interacts with the cell cycle or vice versa. These range from traditional approaches toward measuring the cell cycle and virus production to the use of confocal microscopy to investigate the redistribution of cell cycle factors in virus-infected cells to a proteomic analysis of the nucleolus, which has recently been identified as having roles in cell cycle regulation (21,22).

2. Materials

2.1. Northern Blot and Detection

1. Ambion BrightStar Psoralen-Biotin kit.
2. Ambion BrightStar BioDetect kit.
3. Ambion NorthernMax Formaldehyde loading dye.
4. Ambion NorthernMax 10X denaturing gel buffer.
5. Ambion NorthernMax 10X MOPS running buffer.
6. Ambion NorthernMax prehybridization/hybridization buffer.
7. 20X Standard saline citrate (SSC) buffer (Invitrogen).
8. 10% Sodium dodecyl sulfate (SDS) solution (Ambion).
9. Ambion BrightStar-Plus positively charged nylon membranes.
10. 3MM blotting paper (Whatman).
11. 1% Neutral red solution in ddH₂O.
12. 2% Low melting point agarose (Sigma) solution in ddH₂O.

2.2. Plaque Assay

1. Vero medium constituents.
 - a. 10X Eagle's Minimal Essential Medium (EMEM; Invitrogen).
 - b. Foetal bovine serum (Invitrogen).
 - c. 200 mM L-Glutamine (Invitrogen).
 - d. 10,000 U/mL Penicillin/10,000 mg/mL streptomycin solution (Invitrogen).
 - e. 7.5% Sodium bicarbonate solution (Invitrogen).
2. Vero medium is made up to a 2X solution to account for dilution with agarose solution. EMEM with 20% foetal bovine serum, 4 mM L-glutamine, 200 U/mL penicillin, 200 µg/mL streptomycin, 3 g/L sodium bicarbonate solution.

2.3. Purification of Nucleoli

1. Chemicals to be used in these procedures should be of the best analytical grade commercially available. When applicable, all solutions should be prepared with double-distilled ion-exchange grade water.
2. All solutions in this nucleolar protocol have been supplemented with Complete Protease inhibitor tablets (Roche, cat. no. 1-873-580) at a final concentration of 1 tablet/50 mL).
3. Phosphate-buffered saline (PBS): 1.15 g/L Na_2HPO_4 , 8 g/L NaCl, 100 mL/L MgCl_2 , 200 g/L KH_2PO_4 , 200 g/L KCl, 132 mL/L CaCl_2 .
4. HEPES: 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl_2 , 0.5 mM dithiothreitol (DTT).
5. The sucrose solutions are made up as follows. S1 solution: 0.25 M sucrose, 10 mM MgCl_2 ; S2 solution: 0.35 M sucrose, 0.5 mM MgCl_2 ; S3 solution: 0.88 M sucrose, 0.5 mM MgCl_2 .

2.4. 2D SDS-PAGE

1. Sample buffer: 8 M urea, 2% 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate, 50 mM dithiothreitol (DTT), 0.2% (w/v) Biolyte 3/10 (Bio-Rad, UK), and a trace of bromophenol blue.
2. Protean IEF cell (Bio-Rad, UK cat. no. 165-4000).
3. 0.1% (w/v) Coomassie brilliant blue solution: 50% methanol, 10% acetic acid, 0.1% (w/v) Coomassie brilliant blue solution (Sigma-Aldrich, UK), 40% water. Store at room temperature. We recommend using the solution only once per gel.
4. Destain buffer: 5% methanol, 7% acetic acid, 88% water. Store at room temperature.
5. IEF gel staining solution (Bio-Rad, UK, cat. no. 161-0434).
6. Equilibration buffer I: 6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 37 mg/mL iodoacetamide, and 2% (w/v) DTT. Alternatively, this can be purchased as cat. no. 163-2107 from Bio-Rad, UK.
7. Equilibration buffer II: 6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol. Alternatively, this can be purchased as cat. no. 163-2108 from Bio-Rad, UK.
8. 1X Tris/glycine/SDS running buffer: prepare a 5X stock solution of SDS-polyacrylamide gel electrophoresis (PAGE) running buffer by mixing 15.1 g Tris base, 72 g glycine, 5 g SDS, and water to 1000 mL. This stock is stable for several weeks; when required dilute it down to 1X for use at the correct pH of 8.3. Alternatively, this stock can be purchased as cat. no. 161-0772 from Bio-Rad, UK.
9. To prepare gels from the second dimension separation, either purchase from Bio-Rad, UK as cat. no. 345-0036 or make them yourself with a suitable gel casting apparatus (based on a 14-cm $\times$ 14-cm $\times$ 0.75-mm cast):
 - a. Briefly, for the stacking gel, 0.65 mL 30% acrylamide: 0.8% *bis*-acrylamide, 1.25 mL 1.5 M Tris-HCl, pH 6.8, 50 μL 10% SDS, 3.05 mL water, 25 μL 10% ammonium persulfate solution, 6 μL TEMED (Sigma-Aldrich, UK).
 - b. Resolving gel, 6 mL 30% acrylamide: 0.8% *bis*-acrylamide, 3.75 mL 1.5 M Tris-HCl, pH 6.8, 150 μL 10% SDS, 5 mL water, 150 μL 10% ammonium persulfate solution, 6 μL TEMED (Sigma-Aldrich, UK).
10. Overlay agarose: 0.5% agarose, 25 mM tris-HCl, 192 mM glycine, 0.1% SDS, and trace bromophenol blue.

3. Methods

3.1. Measuring the Interrelationship Between Viruses and the Cell Cycle

There are two options for investigating the cell cycle stage and affect on virus replication. The first is to synchronize cells using serum starvation, release the cells, and determine the cell cycle profile by labeling the cells with bromodeoxyuridine (BrdU) and propidium iodide and analyzing on a flow cytometer. Cells can then be infected at different stages of the cell cycle; depending on the length of the virus life cycle virus replication can occur within a particular cell cycle stage. The second alternative is to use cell cycle inhibitors to block a particular stage of the cell cycle. This has the advantage that cells can be inhibited for prolonged lengths of time, perhaps sufficient to cover the infections cycle; it also has several disadvantages, including the fact that not all cells will arrest at the same stage 100% of the time, and cell cycle inhibitors may affect viral processes themselves.

Simple experiments can be performed to investigate whether a virus has an effect on cellular proliferation and/or the cell cycle. For example, a Coulter counter can be used to measure cellular proliferation in infected compared with noninfected cells (**10**) and the cell cycle profiles of infected cells compared with noninfected using flow cytometry (**Fig. 1**). Infection protocols vary from virus to virus, and these can be found by consulting specific literature. Below we detail our protocols for determining the cell cycle stage.

3.1.1. Flow Cytometry

3.1.1.1. HARVESTING OF CELLS FOR FLOW CYTOMETRY

1. To harvest cells for flow cytometry, remove the growth medium and wash the monolayer twice with 3 mL PBS.
2. To detach the cells, add 2 mL of PBS/EDTA/trypsin and incubate at 37°C for 5 min or until cells detach (*see Note 1*).
3. To remove the remaining cells, scrape using a cell scraper (Sarstedt). To inactivate the trypsin, transfer by pipeting into a canonical tube filled with 8 mL 10% DMEM.
4. The suspension is then centrifuged at 250g for 10 min at 4°C, the supernatant is removed, and the cell pellet is resuspended in 2 mL ice cold PBS prior spinning at 250g for 10 min/4°C.
5. The supernatant is removed, and the cells are processed for flow cytometry to detect either incorporated BrdU or cell cycle marker proteins.

3.1.1.2. ANALYSIS OF STAINED CELLS

1. Fixed and stained cells are analyzed by flow cytometry with a fluorescence-activated cell sorter (FACScan; Becton Dickinson; or equivalent).
2. Ten thousand events per sample should be collected, stored, and analyzed using CellQuest software (Becton Dickinson).

Fig. 1. (A) Flow cytometry analysis of mock and avian coronavirus-infected cells at 0 and 16 h post infection. (B) Detection of avian coronavirus protein by indirect immunofluorescence in infected cells. Vero cells were infected with coronavirus for 0 h or 16 h, fixed, and analyzed by indirect immunofluorescence using appropriate antibodies. (Original magnification x620.) The data indicate that infected cells accumulate in S-phase compared with mock infected cells.

3.1.1.3. FLOW CYTOMETRY ANALYSIS OF BrdU INCORPORATION

1. To determine the proportion of cells undergoing DNA replication, cells are pulsed by addition of 10 mM BrdU, an analog of thymidine, for 30 min prior to harvesting.
2. Cells can be harvested by trypsinization and rinsed with PBS as described. After centrifugation at 250g for 10 min at 4°C, the pellet is suspended in 1 mL of precooled (–20°C) 70% ethanol (*see Note 2*).
3. To remove the fix, cells are centrifuged at 300g for 10 min at 4°C and the ethanol removed.
4. To denature the DNA, 500 µL of 0.1 M HCl is added and incubated at 37°C for 15 min.
5. The reaction is then stopped with the addition of 3 mL PBS and centrifugation at 300g for 10 min/4°C.
6. The supernatant can be discarded. Then 100 µL of anti-BrdU staining solution (20 µL anti-BrdU antibody [Becton Dickinson], 80-µL PBS, 0.5% Tween-20, 1% fetal calf serum [FCS]) is added to the nuclei and incubated for 60 min at room temperature.
7. Excess antibody is removed by the addition of 2 mL PBS, and the suspension is centrifuged at 300g for 10 min/4°C.
8. Remove the PBS, replace with 100 µL of goat antimouse fluorescein isothiocyanate (FITC) antibody staining solution (10 µL goat antimouse FITC antibody [Sigma], 90 µL PBS, 0.5% Tween-20, 1% FCS), and incubate for 30 min in the dark.

9. Excess antibody should be removed by adding 2 mL PBS and centrifuging at 300g for 10 min/4°C.
10. For DNA staining, the cells are incubated in 1 mL staining solution (200 µg/mL RNase A [Sigma], 50 µg/mL propidium iodide [Sigma] in a FACS flow device [Becton Dickinson]) for 30 min at room temperature in the dark.
11. Fixed and stained cells are then transferred into FACS tubes (Becton Dickinson) and analyzed by flow cytometry.

3.2. Measuring Virus

There are several stages to the virus life cycle, and these include attachment to the host cell, entry, uncoating of the genome, transcription and translation of viral mRNAs, replication of the genome, packaging of new genomes, virus assembly, and release of new virus particles. Several assays can be used to measure these different stages of virus infection; however, a simple analysis measuring replication and total virus production will provide information as to whether a cell cycle stage affects virus infection. One of the simplest ways to measure virus replication is to use Southern (in the case of DNA viruses) and Northern (in the case of RNA viruses) blots. Overall yields of virus can be determined by plaque assay. Using an RNA virus as an example, we detail two protocols that can be used to measure RNA replication (mRNA production) and amount of virus. These can be readily adapted to study other viruses. We routinely work with coronaviruses, which are positive-strand RNA viruses, and principally cause respiratory infection (e.g., severe acute respiratory syndrome).

3.2.1. Methods for Analyzing Virus Production

3.2.1.1. NORTHERN BLOT

The following nonisotopic Northern blot protocol has been used in our laboratory for the detection and analysis of coronavirus-derived RNA species. RNA is routinely obtained by extraction of cytoplasmic RNA from coronavirus-infected cells using the Qiagen™ mini-prep RNA purification kit. For longer RNA species, or for the preparation of RNA that is free from DNA or from tissues and organs, we use the Promega RNagents® total RNA isolation system coupled with multiple freeze–thaws and homogenization, and routinely include a DNase (RQ1 DNase, Promega) treatment step (*see Note 3*). (An example of what purified RNA looks like by nondenaturing agarose electrophoresis is shown in **Fig. 2A**.) In this latter case, all purification steps are conducted at 4°C (i.e., in a cold room). When handling RNA, particular care should be taken to avoid contamination (*see Note 4*).

3.2.1.1.1 Sample Preparation and Denaturing Agarose Gel Electrophoresis (*see Note 2*).

1. Typically 4–6 µL (approx 5 µg) of each RNA sample are denatured by addition of 3 vol of formaldehyde loading dye, incubation at 65°C for 15 min, and then rapid chilling of samples in a wet-ice bath.
2. Denatured RNA samples are loaded on a 1% formaldehyde denaturing agarose gel, comprised of 90 mL nuclease-free water, 10 mL of 10X formaldehyde denaturing buffer, and 1 g of agarose, within a horizontal submarine gel electrophoresis tank (*see Note 5*).

Fig. 2. (A) RNA extracted from Vero cells using the Promega RNAGents® total RNA isolation system. This is a nondenaturing 1% agarose gel, and the RNA is visualized by staining with ethidium bromide. In good RNA preparations, the ratio of 28s rRNA to 18s rRNA should be approx 2:1. (B) Northern blot analysis of total cytoplasmic RNA extracted from avian coronavirus (IBV B-US)-infected chick kidney (CK) cells. RNA was extracted from IBV B-US-infected CK cells, separated by electrophoresis on a 1% denaturing formaldehyde agarose gel, and transferred to nylon membrane. IBV-derived RNAs were detected by hybridization with an IBV 3' untranslated region genomic probe capable of detecting IBV genomic RNA and IBV mRNAs. The black arrowheads indicate the positions and sizes of the IBV B-US subgenomic mRNAs. The size of the RNA species is indicated in kb.

3. Gels are run in 1X MOPS running buffer overnight (approx 8–16 h) at 30 V (*see Note 6*).

3.2.1.1.2. Capillary Transfer of RNA Species to Membrane

1. Excess agarose gel not containing any potential RNA species is trimmed from the gel and nylon membrane, and approx 10 pieces of 3MM blotting paper are cut to the same size as the gel (*see Note 7*).
2. The upward capillary transfer method is set up as shown in **Fig. 3**. A 3MM paper wick is cut so that it allows transfer of buffer from the reservoir.
3. The 3MM paper wick is presoaked in 20X SSC buffer, and the nylon membrane and three sheets of 3MM paper are presoaked in 2X SSC prior to capillary transfer assembly.

Fig. 3. Diagram showing the arrangement of apparatus for upward capillary transfer for Northern blot. SSC, standard saline citrate.

4. The agarose gel is oriented so that the wells are facing downward to minimize the distance RNA has to migrate through the agarose gel to the nylon membrane.
5. Sufficient dry paper towels must be used draw enough transfer buffer through the gel and membrane to allow efficient transfer to occur. The transfer apparatus is weighed down with a 1-kg weight and left overnight (8–16 h) for transfer to occur.
6. Once transfer is complete, the nylon membrane is washed briefly in 2X SSC buffer, and the RNA is UV-crosslinked (254 nm UV light for 25–50 s) to the nylon membrane (*see Note 8*).

3.2.1.1.3. Blot Probing. In recent years nonradioactive detection of nucleic acid species has been as effective as with radioactive methods. The nonradioactive methodologies have a number of advantages, mainly associated with health and safety issues. We routinely use DNA probes nonisotopically labeled using the Ambion BrightStar Psoralen-Biotin kit. The label is composed of a planer, tricyclic psoralen compound covalently attached to biotin. Psoralen intercalates between nucleic acids and covalently binds during irradiation by long-wave UV light to create biotin-labeled DNA.

1. For each labeling reaction 10 μL of purified DNA template (0.5 ng/ μL –0.5 $\mu\text{g}/\mu\text{L}$) is used, producing five 20 μL DNA-labeled probes. Probes are stored at -80°C .
2. Crosslinked membrane is prehybridized prior to probing in 10 mL pre-hybridization/hybridization solution for 30 min at 42°C within a hybridization tube in a roller oven.
3. Following prehybridization, a 20 μL prepared probe aliquot is diluted to 100 μL with nuclease-free water, incubated at 100°C for 10 min, and then added to the hybridization tube.
4. Membranes are incubated at 37°C overnight (approx 8–16 h). Following hybridization, membranes are washed twice with 20 mL of 2X SSC/1% SDS low-stringency wash solution at 42°C for 5 min and then twice with 20 mL of 0.2X SSC/0.1% SDS high-stringency wash buffer at 42°C for 5 min.

3.2.1.1.4. Probe Detection. Hybridized psoralen-biotin-labeled DNA probe is detected using the Ambion chemiluminescent non-isotopic BrightStar BioDetect kit according to manufacturer's instructions. Hybridized biotinylated DNA probe is bound by a streptavidin/alkaline phosphatase conjugate; the blot is then incubated with detection reagent, resulting in chemiluminescence of labeled probe. Labeled RNA species present on the membrane are then detected following 2–4 h of exposure to photographic film (*see Note 9*). An example of a Northern blot of viral RNA is shown in **Fig. 2B**.

3.2.1.2. QUANTITATIVE PLAQUE ASSAY INFECTIVITY ASSAY TO DETERMINE THE NUMBER OF INFECTIOUS PARTICLES

The following protocol has been successfully used to quantitatively determine the number of infectious avian infectious bronchitis coronavirus particles within a sample by plaque assay using Vero cells within our laboratory. This method can be adapted for calculating the titer of a variety of viruses that induce cytopathic effect in cell culture by adapting the cell type, media, and environmental conditions according to the particular virus. For example, the number of baculovirus infectious particles can be calculated by plaque assay using Grace's insect medium supplemented with FBS on Sf9 cells.

1. An appropriate dilution series of the virus sample is performed, typically in 10-fold dilution steps, for example, addition of 150 μL of neat virus supernatant to 1350 μL of Vero media to make a 10^{-1} dilution, and so on.
2. Aspirate media from Vero cells grown to confluency in 6-well plate dishes. Duplicate infections per dilution are typically performed with 500 μL of inoculum per well of virus.
3. Plates are incubated for 1 h at 37°C within a 5% CO_2 incubator (*see Note 10*).
4. During the 1-h incubation, preheat the Vero media to 37°C .
5. Melt the 2% low melting point agarose solution within a microwave or boiling water bath until the agarose is completely in solution, and then equilibrate to 42°C .
6. At 1 h post infection, aspirate media from cells, and wash twice with PBS.
7. Mix an equal volume of equilibrated media and agarose solution and overlay the cells with 2 mL of solution by carefully pipeting the solution down the side of the well (*see Note 11*).
8. Allow the agarose overlay to solidify fully, and then incubate the plates for 3 d at 37°C within a 5% CO_2 incubator.
9. At 3 d post infection, prepare media/agarose solution and add 1% neutral red stock solution to a final concentration of 0.01% neutral red.
10. Overlay each well with 2 mL of the media/agarose/neutral red solution, and allow to set fully before incubating the plates for 1 d at 37°C within a 5% CO_2 incubator.
11. Plaques can be visualized, on a light box, as clear zones against a red background. The virus titer can then be calculated at a particular dilution as the number of plaque forming units (PFU)/mL (*see Note 12*):

$\text{Titer (PFU/mL)} = \text{average number of plaques} \times 1/\text{vol of inoculum (mL)} \times 1/\text{dilution}.$

For example: an average of 25 plaques from 500 μL of inoculum per infection at a dilution of 10^{-6} .

$\text{Titer (PFU/mL)} = 25 \times 1/0.5 \times 1/10^{-6} = \text{Titer of } 5 \times 10^7 \text{ PFU/mL}$

3.3. Flow Cytometry and Confocal Analysis of Cell Cycle Factors

Viral effects on the cell cycle can often be attributed to interference with cell cycle factors, such as the cyclins. Here we detail our approaches to investigating the effect of virus on what some might consider nonconventional cell cycle factors, caspase 8 and proliferating cell nuclear antigen (PCNA). Viral effects on these factors (as with any factor) should be studied both at the level of their cellular localization, which in turn can affect function, and also for analysis of expression levels in the cell. To study localization we routinely use confocal microscopy, and to study expression levels, we use flow cytometry. Microscopy has the added advantage that it can be used to check that antibodies recognize proteins of interest in both species-specific and -nonspecific cells. Both monoclonal and polyclonal antibodies to various mammalian proteins can be crossreactive depending on the degree of conservation between the various proteins. For example, a monoclonal antibody to fibrillarin (a nucleolar protein) can be used to detect fibrillarin via immunofluorescence in human, monkey, and avian cells (9).

3.3.1. Analysis of Caspase 8

Recent work has identified and highlighted the role of caspases in cell proliferation (23). Caspase 8 has been postulated to activate downstream nuclear caspase 3, which in turn cleave various negative regulators of the cell cycle, such as p21^{Cip1/Waf1} or p27^{Kip} (24), thereby leading to activation of CDK2. Activation of CDK2 in turn induces its dissociation from cyclin E or cyclin A, thereby inactivating cyclin E function by degrading cyclin E by the proteasome pathway and subsequent G₁ arrest; a number of viruses target this protein to usurp its functions.

3.3.1.1. INDIRECT IMMUNOFLUORESCENCE OF CASPASE 8

Cells are grown on cover slips, normally in 6-well plates.

1. Vero cells are either treated or not treated with 100 mM etoposide (Sigma; as a positive control) and left for 48 h. Cells are analyzed for the distribution of caspase 8, following a protocol published in **ref. 25**.
2. Cells are fixed for 20 min at -20°C with 1 mL methanol/acetone (1:1) following removal of growth medium and washing twice with 2 mL PBS.
3. Cover slips are rinsed twice with PBS and permeabilized with 1 mL 0.1% Triton X-100 (Sigma) in PBS for 30 min at room temperature (RT).
4. The solution is then removed and the cover slips rinsed twice with PBS followed by blocking for 1 h at RT with 4% BSA in PBS.
5. The blocking solution is removed, and rabbit polyclonal anticaspase 8 P-20 (cat. no. H-134, Santa Cruz Biotechnology) diluted 1:100 in blocking solution (4% BSA in PBS) is incubated for 4 h at RT.
6. Excess antibody should be removed and the cover slips rinsed twice with PBS before incubating in PBS for 30 min at RT.
7. Secondary FITC-conjugated antirabbit antibody (Sigma) diluted (1:100) in PBS is then incubated for 1 h at 37°C.
8. Excess antibody is removed by washing the cover slips in 2 mL PBS three times for 10 min each prior to mounting the cover slips on microscope slides using mounting media containing DAPI (Vector).

9. Mounted coverslips can be analyzed using an Axiovision system (Carl Zeiss Jena). Pictures are captured with an AxioCam camera and processed using the Axiovision 3.0 software provided (Carl Zeiss Jena).

Figure 4 provides an example of the distribution of caspase 8 in wild-type cells, cells expressing the avian coronavirus nucleoprotein (which we know has cell cycle effects and redistributes nucleolar proteins), and control cells. As can be seen from the figure, caspase 8 is redistributed to the nucleolus in cells expressing a viral protein (IBVNHIS) from an expression plasmid.

3.3.1.2. FLOW CYTOMETRY OF CASPASE 8

1. For flow cytometry of caspase 8 expression, Vero cells either treated with 100 mM Etoposide for 48 h for transfected for 24 h (either with pTriEx 1.1 [Novagen] or pTriEx IBVNHIS) are harvested by detaching the cells with 2 mL PBS/EDTA/trypsin and transferring them into canonical tubes containing 8 mL 10% DMEM.
2. The supernatant containing detached cells is transferred to canonical centrifuge tubes instead of being discarded.
3. In both cases cells are spun at 250g for 10 min at 4°C, and the cell pellets resuspended in 2 mL ice-cold PBS.
4. Prior centrifugation, both populations are pooled, and the pooled cells are then pelleted by centrifugation at 250g for 10 min at 4°C.
5. The supernatant is removed, and the cells are resuspended in 70% precooled (−20°C) methanol and incubated for at least 12 h at −20°C.
6. To detect intracellular caspase 8, cells are centrifuged at 250g for 10 min/4°C to remove methanol and washed once with 2 mL ice-cold PBS.
7. Cells are then resuspended in 875 µL of ice-cold PBS and fixed by the addition of 175 µL of ice-cold PBS with 2% paraformaldehyde, pH 7.4, for 1 h at 4°C.
8. Fixative can be removed by centrifugation for 5 min (250g, 4°C) and the supernatant aspirated.
9. Cells are permeabilized by resuspending them in 1 mL of PBS with 0.05% Tween-20 for 15 min at 37°C; then they are washed with 1 mL of PBS and centrifuged for 8 min at 250g at 4°C.
10. The presence of caspase 8 can be detected by using rabbit polyclonal antibody against caspase 8 P-20 (1:100; Santa Cruz Biotechnology) and goat antirabbit FITC-conjugated antibody (1:100; Sigma).

3.3.2. Analysis of Proliferating Cell Nuclear Antigen

Proliferating cell nuclear antigen (PCNA) expression is associated with S-phase, and its localization is restricted to sites of DNA replication, as shown by immunofluorescence analysis. During DNA replication, PCNA functions as an auxiliary protein for DNA polymerase α , and its presence is necessary for synthesis of the leading strand, although the precise function has not been clarified.

3.3.2.1. INDIRECT IMMUNOFLUORESCENCE ANALYSIS OF PCNA

1. Cells can be grown on glass cover slips and are washed twice with PBS before fixation with 100% precooled (−20°C) methanol at −20°C for 5 min. (In this example we are using Vero and HeLa cells.)

Fig. 4. Localization of caspase 8 in transfected or etoposide-treated Vero cells or cells expressing the avian coronavirus nucleoprotein. Vero cells were transfected with pTriExIBVNHIS (a construct that expresses the nucleoprotein under the control of a PolII promoter), treated with 100 mM etoposide, or left untreated and analyzed by indirect immunofluorescence for the localization of caspase 8 using a polyclonal anticaspase 8 (P 20) antibody (Santa Cruz Biotechnology). Transfected cells were analyzed 24 h post transfection, whereas etoposide-treated cells were analyzed 48 h post treatment. In nontreated cells (left row) caspase 8 can be detected in both the nucleus and the cytoplasm, in cells transfected with IBVNHIS (middle row), caspase 8 is localised in the cyto- and nucleoplasm with a prominent signal in the perinuclear region and the nucleolus. In Etoposide treated cells (right row) caspase 8 is almost exclusively localised in the nucleus and the perinuclear region. Primary caspase 8 antibody was detected with FITC-conjugated goat antirabbit antibody (Sigma), and IBVNHIS was detected with mouse anti-His (C-term; Sigma) antibody as primary antibodies and Texas red-conjugated goat anti mouse (Harlan Sera Lab) as secondary antibody.

Fig. 5. Localization of proliferating cell nuclear antigen (PCNA) in HeLa and Vero cells. HeLa and Vero cells were grown in 10% DMEM medium on glass cover slips and labeled for PCNA using a mouse monoclonal anti-PCNA antibody (Santa Cruz Biotechnology) as primary antibody and FITC-conjugated goat anti PCNA antibody (Sigma) as secondary antibody. DAPI-containing mounting medium (Vector) was used to counterstain DNA. In both HeLa and Vero cells, PCNA was detected exclusively in the nucleoplasm..

2. Cover slips should be air-dried and washed once with 2 mL PBS prior to addition of 3.5% paraformaldehyde in PBS for 30 min at 4°C.
3. To remove the fix, cells are permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 2 min at RT.
4. After extensively washing four times, each time for 10 min, with PBS, the cover slips should be covered with 500 μ L of mouse monoclonal anti-PCNA (PC 10) antibody (1:100 in PBS) and incubated for 1 h at 37°C in a humidified atmosphere.
5. Cover slips are then washed three times in 2 mL PBS and stained with 500 μ L FITC-conjugated secondary goat antimouse (Sigma) antibody (1:100 in PBS).
6. After 1 h of incubation at 37°C, the cover slips are washed three times with 2 mL PBS and mounted using mounting medium containing DAPI (Vectashield, Vector). (Proteins can be visualized as in **Subheading 3.3.1.1., step 9**).

As can be seen in **Fig. 5** antibody to human PCNA can detect this protein in both human (HeLa) and Vero (a monkey cell line) cells. Thus the antibodies can be used to detect proteins in nonspecies-specific cell lines.

Fig. 6. Analysis of PCNA expression in Vero cells expressing the avian coronavirus nucleocapsid proteins or cells infected with the virus IBV B-US. The cell population was gated in two subpopulations: PCNA-negative (R1) and -positive (R2) cells using CellQuest software. Shown are representative dot plots of mock-transfected cells expressing N protein, control, and IBV Beaudette-infected cell populations. Averages of three experiments for infected cells are shown in the accompanying charts.

3.3.2.2. FLOW CYTOMETRY ANALYSIS PCNA

As can be seen in **Fig. 6** the number of cells expressing PCNA in avian coronavirus-infected cells or cells expressing the viral nucleoprotein (when expressed from an expression plasmid) is less than when compared to mock treated cells.

To detect the number of cells expressing PCNA, flow cytometry can be used. Cells were harvested as described above prior to flow cytometry analysis. The protocol used followed essentially a protocol published by **ref. 26** with slight modifications. In our case we have used Vero cells as an example, as these support avian coronavirus infection.

1. Pelleted cells are fixed by the addition of 1 mL 1% paraformaldehyde in PBS and incubated for 2 min at RT.
2. The fix is removed by addition of 2 mL PBS and centrifuging at 250g for 10 min at 4°C. To postfix and permeabilize the cells, the cell pellet was then resuspended in 200 μ L PBS followed by the addition of 2 mL precooled (-20°C) 100% methanol and incubation for 5 min at -20°C . The fix was removed by spinning at 250g for 10 min at 4°C.
3. The cell pellet is then resuspended in 100 μ L of primary antibody (mouse monoclonal anti-PCNA (PC-10, Santa Cruz Biotechnology) diluted 1:100 PBS and incubated for 1 h at RT.
4. To remove excess antibody, 2 mL of ice-cold PBS are added and the suspension centrifuged at 250g for 10 min/4°C.
5. FITC-conjugated goat antimouse secondary antibody is added in a dilution of 1:100 in PBS and incubated for 1 h in the dark at RT.
6. Excess antibody is removed by adding 2 mL of blocking solution and spinning at 250g for 10 min/4°C.
7. To stain the DNA, resuspend the cell pellet in 1 mL propidium iodide staining solution (50 $\mu\text{g}/\text{mL}$ propidium iodide [Sigma] and 200 $\mu\text{g}/\text{mL}$ RNase [Sigma] in PBS), transfer into FACS tubes (Becton Dickinson), and incubate for 30 min in the dark at RT.
8. Fixed and stained cells are transferred into FACS tubes (Becton Dickinson) and analyzed.

As can be seen in **Fig. 5** the number of cells expressing PCNA in avian coronavirus-infected cells or cells expressing the viral nucleoprotein (when expressed from an expression plasmid) is less than when compared with mock treated cells.

3.4. Proteomic Analysis of the Nucleolus

The following protocols are based on original procedures used by Anderson et al. (27) for the isolation of nucleoli from cultured cell lines. The methods presented here have been successfully used in our laboratory for isolation of nucleoli for subsequent proteomic analysis by 2D SDS-PAGE. This latter method can be used, in conjunction with mass spectrometry, N-terminal protein sequencing, or Western blotting, to identify proteins isolated from the nucleoli. As discussed in the Introduction, the nucleolus is targeted by many different types of virus (13,14), and such interactions may cause perturbations to the distribution of cell cycle factors (e.g., **Fig. 4**) and the cell cycle.

3.4.1. Preparation of Tissue Homogenates and Nucleolar Fractions

1. Prior to harvesting cultured cells for the isolation of nucleoli, rinse each flask three times with prewarmed PBS.
2. On the final rinse step, depending on the cell line, trypsinize off the cells by adding 2–5 mL of trypsin/EDTA solution (Invitrogen, UK) per flask, scrape off with a cell disperser, or bang the flask with a sharp vigorous blow to detach cells. In the former case, swirl the flask and return to the incubator for 3–5 min until most cells have detached.
3. Check that cells are detached using phase contrast microscopy. If cells remain attached to the plasticware, either bang the flask again or increase the trypsin/EDTA incubation period.
4. Once most of the cells are detached, add a volume of culture media at incubator temperature and pipet up and down so that a single-cell suspension is generated. This suspension can now be placed into Falcon tubes for further steps.

5. To wash the cell suspension, spin samples using a swingout rotor at 220g relative centrifugal force (RCF; e.g., rotor A-4-62 Eppendorf, 220g RCF, 1046 rpm), remove supernatant so that a cell pellet remains, and add a volume of 4°C PBS. Carry out this process a further two times before resuspending cells in HEPES (*see Subheading 2.3., item 4 and see Note 13*).
6. Transfer the cell suspension to a precooled tissue homogenizer and homogenize 10 times using a tight-fitting pestle (0.0010–0.0030-inch clearance), while keeping the homogenizer on ice. The number of strokes needed depends on the cell type used, so it is necessary to examine the homogenized cells with a phase contrast microscope after every 10 strokes. Stop when >90% of the cells have burst, leaving intact nuclei.
7. To obtain a pellet containing enriched nuclei, centrifuge the homogenized cells again at 220g for 5 min at 4°C.
8. Resuspend the pellet with a volume S1 solution by pipeting up and down. In another tube containing a volume of S2, layer onto the top the resuspended pellet; ensure that the two layers remain cleanly separated.
9. Centrifuge this cushion at 1430g for 5 min at 4°C to obtain a purer nuclear pellet. Following this spin, discard the supernatant, and resuspend the pellet in a volume of S2 solution by pipeting up and down.
10. Appropriate sonication on ice is a crucial stage in the preparation of nucleoli (*see Note 14*).
11. To prepare a nucleolar concentrated pellet, layer the sonicated material over a volume of S3 solution and centrifuge at 3000 g for 10 min at 4°C.
12. Further purification of nucleoli can be carried out by re-suspending the pellet with S2 solution, and centrifuging at 1430g for 5 min at 4°C. The resulting pellet contains highly purified nucleoli, which can be examined by microscopy and, if required, stored at –80°C.

3.4.2. 2D SDS-PAGE

The use of 2D PAGE has come into widespread usage since the publication of methods combining isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension. Three separate papers by O'Farrell and others have demonstrated that it was possible to combine IEF with gradient SDS-PAGE gels to separate and reveal proteins better in a gel, thus improving the resolution of 1D SDS-PAGE. Two-dimensional SDS-PAGE is particularly useful for the separation of extremely complex protein mixtures.

3.4.2.1. SAMPLE PREPARATION FOR 2D SDS-PAGE

The following protocols are based on equipment and materials available from Bio-Rad.

1. Make up prepared nucleoli to 2.5–3 mg total protein in sample buffer.
2. Remove the desired number of pH 4.0–7.0 ReadyStrip IPG strips (Bio-Rad, UK) from the –20°C freezer, and set them aside to defrost. It is good practice to run two sets of strips per sample, one to be stained following the IEF phase, and the other to be used for the 2D and subsequent analysis.
3. Using a suitable tray, place sufficient sample volume into each well so that each IPG strip is in contact with the solution through its entire length. Lay the IPG strip gel side down into the sample buffer. Ensure that all samples are evenly in contact with the strip since it

will not be absorbed through the plastic backing. It is recommended that for strips of 11 cm, 185 μ L of sample be used per strip (approx 250 μ g per strip). When preparing samples, do not place the vials on ice, as the urea will crystallize out of the solution.

4. Leave these strips for 1 hr, then overlay each of the strips with 2–3 mL of mineral oil to prevent evaporation during the strip rehydration process.
5. For rehydration cover the tray with a lid and leave the tray sitting on a level bench overnight (11–16 h) to rehydrate the IPG strips thoroughly with the nucleolar sample; rehydration is crucial to successful 2D.

3.4.2.2. ISOELECTRIC FOCUSING.

1. In the IEF tray (Bio-Rad, UK; see manufacturer's instructions), place a paper wick at both ends of the channels covering the wire electrodes. Pipet 8 μ L of pure water onto each wick to wet them.
2. Following strip rehydration, remove strips from the incubation tray using forceps, carefully holding the strips at the end where there is no gel, and hold the strip vertically for 7–8 s until the mineral oil has drained. Each strip can then be transferred to the corresponding channel in the focusing tray, gel side down, with the positive end of the strip adjacent to the positive electrode of the tray, in contact with the wetted electrodes.
3. Each of the strips should again be covered with 2–3 mL of fresh mineral oil.
4. The focusing tray can now be placed into the protean IEF cell (Bio-Rad, UK) with the positive side of the tray corresponding to the positive electrode of the cell.
5. Once the cell cover has been closed, the IEF cell can be programmed (*see* Bio-Rad Protean IEF cell instructions) for a single- or multiple-step focusing protocol. In our laboratory a three-step protocol has proved satisfactory for IEF of nucleolar proteins. Our program follows the basic format of:

Step 1 linear voltage ramp, 250 V for 20 min.

Step 2 linear voltage ramp, 8000 V for 2.5 h.

Step 3 rapid voltage ramp, 20,000 VHrs.

6. When setting up the program, it is satisfactory to use the default IEF cell temperature of 20°C, with a maximum current of 50 μ A per strip. This three-step program takes approx 6 h. Once the program has finished, it is important either to place the strips under a 500-V holding voltage or remove, cover with tin foil, and freeze at –80°C or stain for protein.

3.4.2.2.1. Staining IPG Strips With IEF Stain or Coomassie Stain

1. For determining whether proteins have isoelectrically focused correctly, it is possible to transfer IPG strips to a clean, dry piece of blotting filter paper with the gel side up, thoroughly wet a second filter paper of the same size with pure water, and carefully lay the wet filter paper onto the IPG strips. Then “peel” back the top filter paper. This blotting step removes mineral oil on the surface of the IPG.
2. The IPG strips can then be stained for protein using 0.1% (w/v) Coomassie brilliant blue solution (1 h) and then destained with destain buffer until proteins are revealed (1–3 h; *see Subheading 2.4.*). Alternatively, strips can be stained with Bio-Rad's IEF stain (Bio-Rad, UK).

3.4.2.3. SEPARATING SAMPLES IN THE SECOND DIMENSION

1. If strips were frozen following the first dimension separation, then remove from the –80°C freezer and allow to defrost thoroughly. It is best to not leave the thawed IPG strips for longer than 15–20 min, as diffusion of the proteins can result in reduced sharpness of the protein spots.

2. Take either the thawed or the freshly prepared strips and place each in an incubation tray with 4 mL of equilibration buffer I for 10 min on a slowly rotating orbital shaker. At the end of the 10-min incubation, discard the used equilibration buffer I in its entirety by carefully decanting the liquid from the tray. To each strip then add 4 mL of equilibration buffer II, and place on an orbital shaker for a further 10 min.
3. During this incubation either prepare SDS-PAGE gels and ensure that the stacking gel is of sufficient size to take the IPG strip, or obtain a suitable number of precast polyacrylamide gels for your samples (*see Subheading 2.4.*).
4. Once the combs have been removed from the gels, rinse each well briefly with nanopure water using a water bottle. Working quickly, prepare sufficient 1X Tris-HCl/glycine/SDS running buffer to run the number of gels you have decided upon.
5. Once the strips have finished incubating and the gels are prepared, melt sufficient overlay agarose in a microwave to cover the IPG strips once they are inserted into the wells.

3.4.2.4. RUNNING THE IPG STRIPS IN THE SECOND DIMENSION

1. Ensure that the IPG wells of the gels are free of any liquid by blotting with Whatman 3MM paper.
2. Remove the incubated strip from the incubation tray, and dip each IPG strip into a tube of suitable length to take the entire length containing 1X Tris-HCl/glycine/SDS running buffer.
3. Carefully lay each strip gel side up and onto the back plate of the SDS-PAGE gel above the IPG well, and pipet into the well the liquid overlay agarose. Once the well is full, gently move the IPG strip down until it is in contact with the top of the SDS-PAGE gel (avoid trapping any air bubbles).
4. Allow the agarose to solidify for 5 min before proceeding.
5. Once the agarose has solidified, mount the gel, fill the reservoirs with running buffer, and begin the electrophoresis, run at the appropriate voltage for the gel size used (*see manufacturer's instructions*; or 150 V for a 14-cm 12% Tris-HCl SDS-PAGE gel).

3.4.2.5. REVEALING PROTEINS IN 2D GEL

1. Once the sample front has reach the bottom of the gel, you can proceed to reveal the proteins in the nucleolar sample by using commercially available stains such as Coomassie blue (*see Subheading 2.*) or silver stain plus (Bio-Rad, UK; carry out staining as recommended by the manufacturer) (**Fig. 7**). If you wish to examine individual, known proteins, the gel can simply be blotted onto nitrocellulose or polyvinyl idene difluoride membranes for probing with specific antibodies. For further information on this latter technique of western blotting, *see ref. 33*.

4. Notes

1. This time will vary depending on the cell type and passage number.
2. Fixed samples can be kept at 4°C until used.
3. DNase should be removed prior to any cloning by reverse transcriptase (RT)-PCR. A phenol/chloroform extraction followed by ethanol precipitation is suitable for this.
4. To avoid RNase contamination, at all stages it is highly advantageous to use either nuclease-free or diethyl pyrocarbonate (DEPC)-treated water to make up all solutions. All work areas and equipment should be cleaned with an RNase inhibitor (such as Ambion RNaseZap), and gloves should be worn at all times. It is also advisable to use preracked

Fig. 7. Silver-stained 2D gel of purified nucleoli focused in the first dimension using a 3-10 strip (Bio-Rad) and then subsequently subjected to electrophoresis on a 10–20% SDS-PAGE gel.

RNase-free tips and prepacked RNase-free tubes. When conducting RNA work, ensure that all liquid dispensers (i.e., Gilson pipets or equivalents) are wiped with 75% ethanol, and wear suitable gloves. Be sure not to touch any part of exposed skin (i.e., your face) with the gloves; this is a bad habit that many laboratory workers have. Many people secrete DNase and RNases.

5. It is advisable to use as thin a gel as possible; the thinner the gel, the faster and more efficient the transfer of RNA to membrane.
6. Running agarose gels for Northern blot analysis at high voltages can result in gel warping owing to heat. It is advisable to run the gel overnight at low voltages to minimize temperatures, and it is advantageous to use a buffer recirculation pump.
7. Touch the nylon membrane as little as possible to prevent nuclease contamination.
8. Once they are crosslinked, membranes can be stored at -20°C for several months.
9. Unlike isotopic ^{32}P -labeled probes, placing the film cassette containing the membrane exposed to film at -80°C will not increase the signal of weak RNA-labeled species.
10. Every 15 min, gently agitate the plates to ensure that the Vero cells are fully overlaid with inoculum to prevent cell desiccation.
11. Ensure that the agarose is fully equilibrated to 42°C before addition to the media. Addition of agarose solution at temperatures higher than 42°C can damage the cells.
12. Virus titer can vary depending on the cell type used.
13. It is good practice to ensure that cells have not lysed but have become swollen in the buffer conditions by examining with an inverted microscope. Extra care should be taken when working with mammalian cells, as these are particularly prone to lysis at 37°C in hypotonic conditions; therefore preparation on ice is imperative.
14. With most cell preparations, sonication of the nuclear suspension for six 10-s bursts (with 10-s intervals between each burst) has proved sufficient in our hands. In our laboratory we use a Misonix XL 2020 sonicator fitted with a microtip probe set at a power setting of 5. The optimal sonication conditions do, however, depend on the cell types used; oversonication leads to destruction of nucleoli, whereas undersonication leaves the sub-

cellular component intact. For best results, examine the suspension by microscopy after each round of sonication; there should be virtually no intact cells, and the nucleoli should be seen as dense, refractive bodies.

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