Productive Influenza Virus Infection of Synchronized Chick Embryo Fibroblast Cells

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SUMMARY

The effects of cell metabolic activity on the outcome of influenza virus infection were studied in partially synchronized chick embryo fibroblast cultures. There was no evidence to show that the time in the cell cycle at which cells were infected had any significant effect on the final virus yield. However, some differences were detected in the length of the latent period between infections established in synchronized or in stationary cells. Influenza virus could replicate in synchronized or normal cell cultures in which DNA synthesis was inhibited with 9-β-d-arabinofuranosyladenine (ara-A).

INTRODUCTION

Influenza virus, in contrast to other negative strand viruses, is unable to initiate multiplication in a cell which lacks a functional nucleus (Cheyne & White, 1969; Follett et al. 1974; Kelly et al. 1974; Pringle, 1977). Although the precise reasons for this are not yet understood, one function provided by the nucleus which is undoubtedly required for influenza virus replication is the activity of the enzyme DNA-dependent RNA polymerase form II. This was first suggested by inhibitor studies which showed that DNA transcription, but not DNA synthesis, is necessary at an early stage in the virus replication cycle (Barry et al. 1962; Barry, 1964; Rott et al. 1965; White et al. 1965). Subsequently it was found that influenza virus replication is strongly inhibited by α-amanitin (Rott & Scholtissek, 1970) and that this inhibition occurs because functional host-cell DNA dependent RNA polymerase II is necessary at a stage earlier than the first detectable synthesis of virus-specific polypeptides (Mahy et al. 1972; Lamb & Choppin, 1977; Spooner & Barry, 1977). The activity of DNA-dependent RNA polymerase II increases transiently in influenza virus-infected cells, whereas the activity of the form I enzyme decreases (Mahy et al. 1972).

Characterization of the changes in enzyme activities and in the rates of DNA and RNA synthesis during the cell cycle have shown that DNA synthesis is largely restricted to the S phase while RNA synthesis tends to precede the peak of DNA synthesis; increases of up to tenfold in the activity of DNA-dependent RNA polymerase II have been observed which correlate with the increased rate of RNA synthesis prior to the S phase (Mitchison, 1969; Ward & Plagemann, 1969; Johnson et al. 1974). Such changes in the activity of DNA-dependent RNA polymerase II and in the rates of DNA and RNA synthesis during the cell cycle might be expected to influence the ability of influenza virus to initiate infection. The experiments described here were designed to investigate the effect of the metabolic status of the host cell at the time of virus infection on the ability of the virus to establish and undergo a productive cycle of replication.
METHODS

Cell culture. Primary chick embryo fibroblasts (CEF) were prepared as described by Borland & Mahy (1968) and grown on 16 x 18 mm pieces of Melinex (Boyden Data Papers, Croydon, Surrey) contained in plastic Repli boxes (Sterilin Ltd, Teddington, Middlesex) in medium 199 (M199) containing 10% calf serum.

Virus. Influenza virus A/FPV/Rostock/34 (fowl plague virus; FPV) was grown in 11-day-old embryonated eggs and infections were carried out using freshly harvested virus-infected allantoic fluid diluted with PBS to give a multiplicity of infection of approx. 50 p.f.u./cell. Virus adsorption was for 30 min at room temperature; after removal of the virus inoculum, cell monolayers were washed four times with PBS before addition of medium and incubation at 37°C.

Radioisotope labelling. DNA synthesis was measured in cultures by pulse-labelling with 1 µCi/ml ³H-thymidine for 30 min at 37°C in M199 containing the appropriate serum concentration. RNA synthesis was measured by pulse-labelling with 5 µCi/ml ³H-adenosine for 20 min at 37°C in the appropriate medium. At the end of the labelling period the cultures were washed with ice-cold PBS and then overlaid with 0.5 N-TCA and left at 4°C for 30 min. The cultures were then rinsed in PBS, fixed in acetone and allowed to air-dry before being placed in toluene scintillant for counting. Radioisotope labelling of cultures grown on 5 cm dishes was carried out as above and samples were processed for determination of TCA precipitable radioactivity by mixing with 0.5% SDS, precipitating with 1 N-TCA and collecting the precipitate on glass fibre discs. Samples were washed three times with 5 ml of 0.5 N-TCA, one with 5 ml of absolute ethanol, air dried and then placed in toluene scintillant for radioactivity counting.

In situ polymerase assay. Cells which were to be assayed for DNA-dependent RNA polymerase II activity were grown on Melinex plastic strips as described in the text. At the appropriate experimental time, the sample strip was removed from the culture, rinsed in PBS, and then fixed for 5 min in a 1:1 mixture of ethanol and acetone at 4°C. The fixed cells were air-dried and stored at 4°C in the presence of anhydrous CaCl₂.

Samples were assayed using a reaction mixture based on that described by Moore & Ringertz (1973) incorporating 0.4 M (NH₄)₂SO₄. Five hundred µl reaction mixture contained 50 µmol tris-HCl, pH 7.9; 6 µmol 2-mercaptoethanol; 75 µmol sucrose; 2 µmol MgCl₂; 1 µmol MnCl₂; 300 nmol each of ATP, GTP and CTP; 200 µmol (NH₄)₂SO₄ and 50 µCi ³H-UTP (52 Ci/nmol). Each strip was incubated with 40 µl reaction mixture for 30 min at 37°C and the reaction was terminated by addition to each sample of ice-cold PBS. The cell samples were then washed six times with ice-cold PBS and subsequently fixed at 4°C for 30 min in a 9:1 mixture of methanol and acetic acid. Following fixation, samples were mounted on glass slides and immersed in 0.5 N-TCA for 60 min at 4°C. After extensive washing with water the slides were prepared for autoradiography as described by Armstrong & Barry (1974). Exposure was for 5 weeks at 4°C. After development, the slides were stained with methylene blue and grain counts were examined under oil immersion using a Vickers Photoplan microscope.

Assay of virus. Virus growth was monitored by the assay of infected culture media for haemagglutinating (HA) activity or by plaque titration of infected media on CEF monolayers (Borland & Mahy, 1968).

Synchronization of primary cultures of CEF cells. Primary cultures of CEF cells were established in M199 containing 10% calf serum. After 24 h, the medium was replaced with M199 containing no serum and incubation continued for 48 h, when the medium
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was again changed to fresh M199 with no serum. Release of cultures from the block in G1 was achieved after a further 12 h by the addition of M199 containing 3% calf serum (CS) and 3% foetal calf serum (FCS). These conditions of stimulation are as described by Humphries & Temin (1974).

Release of starved cultures from the stationary phase, after serum addition, was monitored by the measurement of DNA synthesis.

Chemicals. 9-β-d-arabinofuranosyladenine (ara-A) was obtained from Calbiochem Ltd., Bishop’s Stortford, Herts, England. 3H-thymidine (sp. act. 22 to 27 Ci/mmol), 2-3H-adenosine (sp. act. 20 to 30 Ci/mmol) and 3H-5-uridine triphosphate (42 to 52 Ci/mmol) were obtained from the Radiochemical Centre Ltd, Amersham, Bucks, England.

RESULTS

Synchronization of primary cultures of CEF cells

Cultures which had been synchronized by the procedure described in Methods were pulse-labelled, at 2 h intervals throughout the experiment, with 3H-thymidine (1 μCi/ml) and the radioactivity incorporated into TCA-precipitated material was measured by liquid scintillation counting. The results are shown in Fig. 1(a). There was a lag period of about 8 h before the onset of DNA synthesis which reached a maximum at 19 h after serum addition. In a parallel set of stationary cells, not stimulated by serum, DNA synthesis remained at a low level. A consideration of nine separate experiments showed that the lag period, observed prior to stimulation of DNA synthesis, varied from 4 to 8 h post-serum addition (p.s.a.), with the peak of DNA synthesis occurring at about 18 h p.s.a.; stimulation of DNA synthesis was, on average, some 47-fold that at 8 h p.s.a. In addition, to estimate the degree of synchronization achieved by this method, cells were labelled with 3H-thymidine for 30 min, washed in TCA at 4 °C, fixed in methanol/acetic acid and processed for autoradiography (data not shown). The percentage of total cells which were labelled with 3H-thymidine was determined by counting under the microscope. At the time of maximum DNA synthesis as measured by incorporation of radioactivity into TCA-precipitable material and liquid scintillation counting, autoradiography showed that 61% of the cells were synthesizing DNA in synchronized cultures compared with less than 5% in stationary cells (data not shown).

RNA synthesis was measured in a further set of synchronized cell cultures using a 20 min labelling period with 3H-adenosine (5 μCi/ml). Adenosine, rather than uridine, was used in order to minimize the effects of variations in nucleoside uptake during the cell cycle (Stambrook & Sisken, 1972). RNA synthesis increased slightly from 0 h p.s.a. to reach a maximum at 10 h p.s.a. after which it declined to the original level (Fig. 1b).

Changes in DNA-dependent RNA polymerase II activity after serum stimulation of the starved cultures were measured in fixed cell samples using an in situ polymerase assay (Moore & Ringertz, 1973). Results of the RNA polymerase assays in uninfected synchronized cells are shown as mean number of grains per uninfected cell nucleus which were observed at different times after serum stimulation of the cells (Fig. 1c). RNA polymerase II activity increased slightly through the cell cycle until 16 h p.s.a. after which it declined, towards the initial level of activity. This result corresponded to that obtained for RNA synthesis as measured by 3H-adenosine incorporation; that is, an increase in RNA synthetic activity preceded the increased DNA synthesis, with a subsequent decline in RNA synthesis around the time of maximum DNA synthesis.
Fig. 1. (a) DNA synthesis was measured in synchronized (●—●) and stationary (O——O) CEF cells by labelling five replicate cultures for each treatment with 1 μCi/ml ³H-thymidine for 30 min; samples were then processed to determine the incorporation of radioactivity into TCA-precipitable material (see Methods). (b) RNA synthesis was measured in synchronized CEF cells by labelling five replicate cultures of CEF cells at 2 h intervals following serum addition, with 5 μCi/ml ³H-adenosine for 20 min; radioactivity incorporated into TCA-precipitable material (□——□) was measured as described in Methods. DNA synthesis was monitored in two parallel sets of cultures (●—●). (c) DNA-dependent RNA polymerase II activity (Δ——Δ) was measured using an in situ assay. Cell samples were fixed at different times following serum addition and subsequently assayed for polymerase activity using a reaction mixture containing 0.4 M (NH₄)₂SO₄ and Mn²⁺ ions (see Methods). Duplicate samples were examined at each time-point and the number of grains per nucleus were counted in 100 to 150 different cells. DNA synthesis was monitored in a parallel set of cultures (●—●).
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Fig. 2

Influenza virus infection at different times after serum addition

To determine whether there was a time in the cell cycle when a significant difference in the 12 h yield of virus could be detected, an experiment was set up in which infections were initiated, in synchronized cells, at 2 h intervals after serum stimulation. DNA synthesis was measured in five replicate cultures per time-point by labelling with 3H-thymidine. The media were harvested from each infection at 12 h p.i. and samples assayed for HA activity and yield of infectious virus. The five replicates for each time point were individually titrated for HA activity and the remainder of the samples were then pooled and analysed by plaque titration in CEF cells to determine the yield of infectious virus. The results, presented in Fig. 2, show some variation in the 12 h yields of virus harvested from the different infections; however, there appeared to be no significant differences in the 12 h virus yields which might be related to cell cycle activities at the time of infection.

Virus growth in CEF cultures of different metabolic activities

The growth of influenza virus in various types of CEF culture is shown in Fig. 3. Infections were initiated in four types of culture: in stationary cells where the block due to serum starvation had not been released; in normal cell cultures in which cells were set up under normal conditions and used after 24 h growth; in synchronized cells infected at 0 h p.s.a.; and in synchronized cells infected at 12 h p.s.a.

Virus release was detected as early as 4 h p.i. in stationary cells but not until 6 h p.i. in infected synchronized cells. Once initiated, release of virus from synchronized cells proceeded at a faster rate than in stationary cells. The yields of virus by 12 h p.i. were similar in all infected cultures except for those in synchronized cells infected at 0 and 12 h p.s.a. where virus yields were slightly lower. The data presented in Fig. 3 have been pooled from
DNA synthesis was measured in synchronized and stationary cells, by determining the incorporation of $^3$H-thymidine into TCA-precipitable material during a 30 min labelling period at 2 h intervals following serum addition. Virus infections were initiated at 0 and 12 h p.s.a. © --- ©, infected cultures; • --- •, uninfected cultures.

The effect of virus infection on DNA synthesis in these cells was examined in a parallel set of cultures and the results are shown in Fig. 4. In synchronized cells infected at 0 h p.s.a. DNA synthesis remained at a basal level throughout infection and no stimulation in DNA synthesis was detected at about 8 h p.s.a. However, in cells infected at 12 h p.s.a., DNA synthesis was not inhibited substantially during the first 5 h after infection but between 5 and 13 h p.i. (see Fig. 4a) DNA synthesis declined rapidly. Little change was seen in the levels of DNA synthesis in infected, stationary cells (see Fig. 4b).

Influenza virus infection of synchronized or stationary cells resulted in little inhibition of DNA synthesis between 0 h and 5 h p.i.; thereafter DNA synthesis rapidly declined to 5 to 10% that of control cells, by 13 h p.i. The inhibition of DNA synthesis seemed to occur more rapidly in normal cell cultures infected with influenza virus.

**Growth of influenza virus in the presence of ara-A**

The observation that initial growth of influenza virus was slower in synchronized cells infected at 12 h p.s.a. than in stationary, normal or synchronized cells infected at 0 h p.s.a., was investigated further by inhibiting cellular DNA synthesis using ara-A.

Synchronized cultures were established and at 11 h p.s.a. cultures were treated with ara-A at a concentration of $10^{-4}$ M. The cells were subsequently infected at 12 h p.s.a.; ara-A was present throughout virus adsorption and in the medium added to the cultures after infection. DNA synthesis was measured in the cells and the culture medium was assayed for HA activity at intervals following infection.

Fig. 5 (a) shows the pattern of DNA synthesis observed in infected and uninfected syn-
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Fig. 5. (a and b) Ara-A at a final concentration of $10^{-4}$ M was added to cultures of synchronized CEF cells at 11 h p.s.a. At 12 h p.s.a, the cells were either infected with FPV, or mock-infected, in the presence of ara-A. Cultures which were not exposed to ara-A were run in parallel. (a) DNA synthesis was monitored in the different cultures as described in Methods. •—•, Uninfected synchronized cells, without drug; ○—○, infected synchronized cells, without drug; ■—■, uninfected synchronized cells, treated with ara-A; □—□, infected synchronized cells, treated with ara-A. (b) Virus growth was monitored by assaying the culture media at different times following infection for HA activity. ▲—▲, Synchronized cells, without drug; △—△, synchronized cells, treated with ara-A. (c and d) DNA synthesis and virus growth were studied in normal cell cultures (24 h after seeding in M199 2% CS) in the presence and absence of ara-A. (c) •—•, DNA synthesis in uninfected normal cells, without drug; ○—○, infected normal cells, without drug; ■—■, uninfected normal cells, treated with ara-A; □—□, infected normal cells, treated with ara-A; (d) ▲—▲, Virus growth in normal cells, without drug, △—△, normal cells, treated with ara-A.

chronized cells, in the presence and absence of ara-A. DNA synthesis was inhibited by 86% in the ara-A treated cultures, but the inhibitory action lessened with time, possibly due to some division of cells in the cultures causing an effective decrease in drug concentration. The growth of influenza virus in these cultures is presented in Fig. 5(b). In the presence of ara-A, the rate of virus release was increased compared to that in cells where DNA synthesis was not inhibited. To check that this was due to the inhibition of DNA synthesis, and not to a direct effect on the virus itself, infections were set up, in the presence or absence of ara-A, in normal, unsynchronized cultures in which the rate of DNA synthesis was low. The results are shown in Fig. 5(c and d). Release of virus proceeded at the same rate in normal cells whether or not ara-A was present in the cultures.

The experimental data described here lead to the conclusion that a specific time in the
cell cycle is not necessary for the establishment of productive influenza virus infection. However, this conclusion is based on the assumption that the method used here to achieve cell synchrony yielded a cell population which progressed through all the phases of the cell cycle. Stationary cells, released from their metabolic block by serum addition, were stimulated to enter a DNA synthetic phase, but it was not obvious from methylene-blue stained preparations whether these cells went on to divide. Therefore, the possibility that cells undergoing mitosis were not susceptible to influenza virus infections had not been excluded.

**DISCUSSION**

There is now convincing evidence that participation of host cell DNA-dependent RNA polymerase II is necessary for the initiation of influenza virus infection (Mahy *et al.* 1972; Lamb & Choppin, 1977; Spooner & Barry, 1977). Since variations in the activity of host cell RNA polymerases are known to occur during the cell cycle, the experiments described in this paper were undertaken to examine the influence of the host cell cycle on the establishment of influenza virus infection. Previous studies by Long & Cooper (1974) and Lomniczi *et al.* (1977) showed that influenza virus infection of synchronized populations of BHK-21 and CHO-CS4 cells, respectively, could be established irrespective of the time in the cell cycle and that such infections did not differ in the rates of synthesis and amounts synthesized of various virus-specific proteins. However, both these studies used virus-cell systems in which only an abortive cycle of virus replication occurred. Using a productive virus-cell system (Cox *et al.* 1977), evidence has been obtained which suggests that influenza virus multiplication is affected by cell cycle activity. Influenza virus was able to infect and multiply in avian myotubes, fully differentiated cells in which DNA synthesis and cell division have ceased, but not in myoblasts, precursor cells synthesizing DNA and undergoing cell division. Both types of muscle cell were susceptible to infection by a parainfluenza virus (O'Neill & Kendal, 1975).

The virus-cell system used in the present investigation, supports a fully productive infection. The method of synchronization of primary cell cultures yielded a population in which 61% of cells were synthesizing DNA at the peak of the S phase. Both RNA synthesis and DNA-dependent RNA polymerase II activity reached a maximum before the peak of DNA synthesis.

These results suggested that the degree of synchronization was sufficient to detect any differences in virus growth dependent on cell cycle activity. Therefore if an essential function is provided by the host cell early in infection, it must either be present throughout the cell cycle, or be activated by the infecting virus.

Although 12 h yields of virus were similar for infections initiated in both synchronized and stationary cells, virus released into the medium was detected as early as 4 h p.i., in stationary cells but not until 6 h p.i. in synchronized cells. Different durations of latent period have been observed for Rous sarcoma and murine leukaemia virus infections in synchronized chick embryo fibroblast and normal rat kidney cells, respectively (Temin, 1967; Panem & Schauf, 1974). In these systems, the length of the latent period was dependent on the time in the cell cycle at which cells were exposed to virus; infectious virus was released only after mitosis and cell division, presumably due to the requirement for DNA provirus integration during the replication cycle. The longer latent period seen in influenza virus-infected synchronized cells could be due to a number of factors. For example, serum release of cells blocked in G1 may initiate changes in the cell surface membrane which could affect the efficiency of adsorption of virus to synchronized cells. Such changes may also affect the
lipid arrangement in cell membranes which could result in a slower rate of cell-virus membrane fusion and subsequent virus penetration. Finally, virus uncoating and release of input virus RNA within the infected cell might be delayed if synchronized cells contained lower levels than stationary cells of proteases involved in this process.

Infection with influenza (fowl plague) virus strongly inhibits DNA synthesis from 5 h p.i. – the late onset of this inhibition suggests that it is due to cytopathic effects rather than to a direct switch-off of DNA synthesis by a virus function. Infection of synchronized cells at 0 h p.s.a. prevents the onset of the S phase, whereas infection at 12 h p.s.a. allows the continued increase in DNA synthesis until 5 h p.i. Subsequently, an increasing inhibition of DNA synthesis occurs.

Influenza virus was able to initiate infections in cells which were treated with ara-A, and 12 h yields were similar to those in untreated cells, in agreement with previous reports using ara-C and other inhibitors of DNA synthesis (Scholtissek & Rott, 1961; Buthala, 1964; Bader, 1965; Nayak & Rasmussen, 1966). Moreover, in cells infected at 12 h p.s.a. when DNA synthesis was increasing, the amount and rate of virus production was increased by the presence of ara-A before and during infection. These results suggest that the level of DNA synthesis or some associated event in cells early in infection may be of importance in determining the initial rate of virus release. In agreement with this, we found differences in the rates of virus release from cells infected at 0 h compared to 12 h p.s.a. (Fig. 3), which may be related to the levels of DNA synthesis in cells at these times, or to the switch of DNA template from RNA transcription to DNA replication.

In conclusion, our results suggest that distinct phases of the cell cycle have little effect on the ability of FPV to initiate productive infections, but differences in the characteristics of virus growth may exist between infections established in stationary and synchronized cells. Although changes in the activity of DNA-dependent RNA polymerase II clearly occur during the cell cycle, these changes do not significantly affect the outcome of influenza virus infection.

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