The Topography of RNA Synthesis in Cells Infected with Fowl Plague Virus

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SUMMARY

The site of influenza virus-induced RNA synthesis in infected chick embryo cells has been determined by autoradiography. Following 5 min pulses of [³H]-uridine, two distinguishable phases of induced RNA synthesis could be detected by grain counting in the nucleus, both of which occurred predominantly in the nucleoplasm. Cytoplasmic RNA synthesis could not be detected in fowl plague virus (FPV) infected cells; a significant increase in cytoplasmic grain count was detected in NDV-infected cells from 4 to 8 h after infection.

Cordycepin ('3-deoxyadenosine) inhibited nucleolar RNA synthesis in chicken embryo fibroblasts (CEF) to a greater extent than nucleoplasmic RNA synthesis; FPV-induced RNA synthesis in cordycepin-treated cells occurred in the nucleoplasm. α-amanitin treatment of FPV-infected cells inhibited the first peak of virus-induced nucleoplasmic RNA synthesis.

Fixed preparations of whole FPV-infected cells were incubated with an RNA-dependent RNA polymerase reaction mixture and examined by autoradiography. A peak of enzyme activity was detected at 3 h after infection in the nucleoplasm; a second peak of activity was detected at 6 h after infection and was wholly cytoplasmic.

We conclude that RNA synthesis in vivo in cells infected with influenza viruses occurs in the cell nucleus and that the increased level of nucleoplasmic RNA synthesis at approx. 1 h after infection signifies increased transcription of cell DNA. The evidence suggests that the microsomal RNA-dependent RNA polymerase found in FPV-infected cells does not function in vivo.

INTRODUCTION

There is considerable uncertainty concerning the site of virus-induced RNA synthesis in cells infected with influenza viruses. An early report (Scholtissek et al. 1962) suggested that virus particle associated RNA was made in the nucleus. More recently a virus-specific RNA polymerase has been identified as a constituent of the virus (Chow & Simpson, 1971; Penhoet et al. 1971) and a similar enzyme is associated with cytoplasmic elements of infected cells (Ho & Walters, 1966; Ruck et al. 1969; Scholtissek & Rott, 1969; Skehel & Burke, 1969; Mahy & Bromley, 1970). Cell fractionation studies reveal the presence of newly synthesized virus RNA in both the cytoplasm and nucleoplasm, but RNA complementary to virus particle RNA is predominantly cytoplasmic (Krug, 1972).

There is evidence to suggest that nuclei play an essential role at least in the initiation of
influenza virus-induced RNA synthesis. Virus components cannot be synthesized in enucleated cell fragments (Cheyne & White, 1969) or in cells in which DNA function has been inhibited by actinomycin D or u.v. irradiation (Barry, 1964; White & Cheyne, 1965, 1966); para-influenza virus replication is unaffected either by enucleation or inhibitor treatment. Virus replication is sensitive in its early stages to α-amanitin (Mahy, Hastie & Armstrong, 1972), a finding that suggests cell gene transcription may be involved in the early stages of infection. It has also been shown that RNA-dependent RNA polymerase accumulates in cell nuclei before its detection in the cytoplasm (Hastie & Mahy, 1973). Circumstantial evidence such as this suggests that the nucleus may be at least one of the sites of virus-induced RNA synthesis.

In this paper we report an autoradiographic study of the time course of RNA synthesis in cultured chick embryo cells infected with fowl plague virus. The object of the study was to detect the site(s) of virus-induced RNA synthesis before any appreciable intracellular transport of RNA occurs, using a method that avoids cell fractionation. Accordingly, infected chick cells received short pulses of [3H]-uridine at half hourly intervals throughout the growth cycle, and the number and topographical distribution of grains was observed. The same technique has also been employed to examine the effects of the inhibitors of RNA synthesis, α-amanitin and 3′ deoxyadenosine (cordycepin), on cellular and virus-induced RNA synthesis. Finally, we report on the in situ detection of RNA-dependent RNA polymerase activity in virus-infected cells, using the technique of Moore & Ringertz (1973).

METHODS

Materials. 3′-deoxyadenosine (cordycepin) was purchased from the Sigma Chemical Company, London, and was used as a freshly prepared solution in maintenance medium (50 μg/ml). α-amanitin (Boehringer Ingelheim Ltd) was used at a concentration of 20 μg/ml. [3H]-uridine (27.6 Ci/mmol) and [3H]-UTP (11.7 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks.

Growth of cells. Primary chick embryo fibroblasts (CEF), prepared as described previously (Borland & Mahy, 1968), were grown on 16 × 18 mm Melinex pieces in plastic Repli dishes, in 1 ml 199 medium containing 10% calf serum. Cells were maintained in medium containing 2% calf serum after infection.

Growth of viruses. The viruses A/FPV/Rostock/34 (fowl plague virus) and Newcastle disease virus, Texas strain (NDV), were used in the form of infected allantoic fluid from embryonated eggs. For infection, cells received an input of approx. 20 p.f.u./cell. The adsorption period consisted of 30 min incubation at 37°C. Control cells received a comparable dilution of uninfected allantoic fluid.

Autoradiography. Cells received 5 μCi/ml [3H]-uridine as a 0.02 ml drop 5 min prior to fixation. Each time point was determined with two or three replicate Melinex strips, which were rinsed in cold PBS and fixed with two changes of a 9:1 mixture of methanol and acetic acid. After rinsing in methanol, each sheet of cells was mounted on a labelled slide, washed in tap water for 30 min and allowed to dry. Slides were coated with Ilford K5 research emulsion, previously melted and diluted 1 in 5 in distilled water at 45°C and allowed to cool to room temperature. They were then stored in a dry, light tight box at 4°C for 3 weeks, and developed in Kodak D19 developer (10°C for 5 min), and fixed with Hypam (1/5 for 4 min). Each preparation was stained with methylene blue, covered with DPx mountant and a coverslip, and examined under oil immersion. Individual grains could be distinguished in cytoplasm, nucleus and nucleolus of all cells. Grain counts for at least 50 cells were
Fig. 1. Autoradiography of uninfected, FPV- and NDV-infected CEF cells. Primary cultures of CEF cells were grown on 16 x 18 mm Melinex plastic strips. Semi-confluent cultures were infected with either FPV or NDV, and at half-hourly intervals pairs of cultures received 20 μCi/ml [3H]-uridine (27.6 Ci/mmol) for 10 min, and then immediately prepared for autoradiography. (a) Control cells at 4 h from beginning of experiment. (b) FPV-infected cells 4 h p.i. (c) NDV-infected cells at 8 h p.i.

obtained for each replicate at all time points in individual experiments, and average values obtained.

*In situ* polymerase activity. This method is a modification of that developed by Moore & Ringertz (1973). Cells were grown on Melinex strips, and infected with FPV, as described above. At each time point, cells were fixed in cold absolute ethanol:acetone (1:1) for 5 min at 4 °C, dried in air and stored at 4 °C in the presence of CaCl₂.

Cell strips were incubated with 0.02 ml of reaction mixture at 32 °C for 45 min. They were then rinsed with cold PBS, fixed with methanol:acetic acid (9:1), rinsed in methanol, mounted on slides and immersed in 0.5 N-trichloracetic acid for 30 min × 2. After extensive washing with water, the slides were prepared for autoradiography as described above, and stored for 4 to 6 weeks, then developed.

The reaction mixture contained, in a vol. of 0.5 ml: 300 nmol each of ATP, GTP and CTP, 35 nmol [3H]-UTP (11.7 Ci/mmol); 25 μmol mercaptoethanol; 160 μM-tris-HCl buffer, pH 8.4; 1 μM-MnCl₂; 3 μM-MgCl₂; 150 μM-NaCl and 10 μg actinomycin D.
Table I. Cytoplasmic RNA synthesis in infected and uninfected chick embryo cells

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Uninfected</th>
<th>Uninfected</th>
<th>FPV-infected</th>
<th>NDV-infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s.d.</td>
<td>s.d.</td>
<td>s.d.</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>s.d.</td>
<td>s.d.</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.48</td>
<td>2.30</td>
<td>4.82</td>
<td>2.92</td>
</tr>
<tr>
<td>6</td>
<td>3.61</td>
<td>1.92</td>
<td>3.55</td>
<td>2.23</td>
</tr>
<tr>
<td>8</td>
<td>4.04</td>
<td>2.56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* $\bar{x}$ = mean number of cytoplasmic grains per cell for 100 cells.
† s.d. = standard deviation.
‡ $P$ = the probability determined by Student's $t$ test,

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\text{s.e.} \bar{x}_1 + \text{s.e.} \bar{x}_2}$$

that differences arise by chance. s.e.$\bar{x}$ = standard error of the mean.

Cells were infected with either FPV or NDV (20 infectious units/cell) and at 4, 6 or 8 h post-infection were pulse labelled for 10 min with 20 $\mu$Ci/ml $[\text{H}]$-uridine (40 Ci/mmol). After labelling, cells were fixed in methanol-acetic acid, 9:1, for 30 min. For autoradiography, cells were coated with Ilford K5 research emulsion, stored 3 weeks at 4 °C then developed and stained with methylene blue.

RESULTS

Autoradiography of virus-infected cells

In a preliminary set of experiments, designed to test the sensitivity of autoradiography as a means of detecting cytoplasmic RNA synthesis, CEF cells were infected with either FPV or NDV. At intervals following infection, the cells were exposed to 20 $\mu$Ci/ml $[\text{H}]$-uridine for 10 min, and immediately prepared for autoradiography. The typical appearance of cells is shown in Fig. 1. Cells infected with FPV are apparently indistinguishable from controls; both contain heavily labelled nuclei, and virtually no cytoplasmic grains. In this type of experiment we have never found any increase in the number of cytoplasmic grains in influenza virus-infected cells at any stage of the growth cycle. With NDV, a highly significant increase in cytoplasmic grain count occurs during the course of infection (Fig. 1 c, Table I). These experiments indicate that cytoplasmic RNA synthesis can be detected by pulse labelling and autoradiography, and that in the case of NDV-infected cells RNA synthesis is a cytoplasmic event.

RNA synthesis in cells infected with influenza virus

Influenza virus-induced RNA synthesis in whole cells, measured as TCA insoluble radioactivity after sequential pulses of $[\text{H}]$-uridine, occurs in two distinguishable phases represented by peaks of activity occurring at approx. 1 and 3 h following infection (Borland & Mahy, 1968). In an attempt to identify the whereabouts of this synthesis, two sets of CEF cells, one infected and the other control, received pulses of $[\text{H}]$-uridine (5 $\mu$Ci/ml for 5 min) at half-hourly intervals and were prepared for autoradiography. The results are shown in Table 2, and demonstrate that increased levels of RNA synthesis occurred in the nucleoplasm of infected cells at 1 h and between 3 and 4 h following infection. The pattern of RNA synthesis determined in infected cell nuclei by autoradiography is virtually identical to that observed in whole infected cells by means of liquid scintillation counting.

The effects of cordycepin

We have reported recently (Mahy et al. 1973) that the multiplication of influenza virus in CEF is unaffected by doses of cordycepin that suppress total cell RNA synthesis by about
RNA synthesis in FPV-infected cells

Table 2. The synthesis of RNA in the nucleoplasm of chick embryo cells infected with fowl plague virus measured by autoradiography

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Uninfected</th>
<th>FPV-infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>s.d.</td>
</tr>
<tr>
<td>0·5</td>
<td>19·7</td>
<td>8·7</td>
</tr>
<tr>
<td>1·0</td>
<td>12·4</td>
<td>8·9</td>
</tr>
<tr>
<td>1·5</td>
<td>20·9</td>
<td>13·9</td>
</tr>
<tr>
<td>2·0</td>
<td>20·2</td>
<td>14·4</td>
</tr>
<tr>
<td>2·5</td>
<td>26·5</td>
<td>13·5</td>
</tr>
<tr>
<td>3·0</td>
<td>8·1</td>
<td>5·7</td>
</tr>
<tr>
<td>4·0</td>
<td>18·6</td>
<td>14·0</td>
</tr>
<tr>
<td>5·0</td>
<td>16·9</td>
<td>10·1</td>
</tr>
</tbody>
</table>

* † ‡ see footnote to Table 1.

Cells were infected with FPV (20 infectious units/cell), and at intervals thereafter were pulse labelled for 5 min with 5 $\mu$Ci/ml [H]-uridine (27·6 Ci/mmol). After labelling, cell sheets were washed in PBS and fixed in methanol:acetic acid (9:1) for 30 min. For autoradiography, cells were coated with Ilford K5 research emulsion, stored 3 weeks at 4 °C then developed and stained with methylene blue. The cell preparations were examined under oil immersion, and the distribution of grain counts in the nucleoplasm of 100 or more cells was determined for both control cells and for cells infected with FPV.

Fig. 2. The effect of cordycepin on nucleolar and nucleoplasmic RNA synthesis in uninfected cells. Primary cultures of CEF cells on Melinex strips received 50 $\mu$g/ml cordycepin at 0 h, and at various times later, duplicate cultures received 5 $\mu$Ci/ml [H]-uridine (27·6 Ci/mmol) for 5 min. The cells were then fixed in a 9:1 mixture of methanol and acetic acid and prepared for autoradiography. Grain counts were determined for both nucleoli and nucleoplasm using 100 to 150 cells for each time point in three different experiments, and the average results are presented as a percentage of controls. Control cells contained 20·8 ± 5·9 nucleoplasmic grains and 5·5 ± 1·35 nucleolar grains.

$\bullet$, nucleoplasmic grains; $\circ$, nucleolar grains.

70%. Cordycepin (3-deoxyadenosine) is an analogue of adenosine that is rapidly taken up by cells and converted to 3-deoxyadenosine triphosphate (Guarino, 1967). Among its effects, it rapidly suppresses the production of ribosomal and transfer RNA (Siev Weinberg & Penman, 1969; Truman & Frederiksen, 1969). Polysome associated mRNA is also inhibited, probably by prevention of post-transcriptional addition of polyriboadenyllic acid, while the synthesis of heterogeneous nucleoplasmic RNA is relatively unaffected.
Fig. 3. The effect of cordycepin on incorporation of [3H]-uridine in FPV-infected CEF cells. Primary cultures of CEF cells on Melinex strips were infected with FPV at an input multiplicity of 20 p.f.u./cell, and treated with 50 μg/ml cordycepin at 0 h. Controls consisted of duplicate CEF cultures treated with cordycepin alone. At half hourly intervals, control and infected cultures received 5 μCi/ml [3H]-uridine (27.6 Ci/mmol) for 5 min. Cells were then fixed and prepared for autoradiography. The results are expressed as % grains incorporated into

- cordycepin treated FPV-infected cells
- cordycepin treated uninfected cells

•—•, nucleoplasmic grains; ○—○, nucleolar grains.

Table 3. The effect of cordycepin on RNA synthesis in the nucleoplasm of chick embryo cells infected with fowl plague virus, measured by autoradiography

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Uninfected + cordycepin</th>
<th>FPV-infected + cordycepin</th>
<th>% FPV + cordycepin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
<td>s.d.</td>
<td>x</td>
</tr>
<tr>
<td>0.5</td>
<td>7.8</td>
<td>5.3</td>
<td>6.6</td>
</tr>
<tr>
<td>1.0</td>
<td>3.2</td>
<td>2.9</td>
<td>4.1</td>
</tr>
<tr>
<td>1.5</td>
<td>4.7</td>
<td>4.6</td>
<td>3.0</td>
</tr>
<tr>
<td>2.0</td>
<td>4.2</td>
<td>3.3</td>
<td>3.8</td>
</tr>
<tr>
<td>2.5</td>
<td>2.3</td>
<td>2.4</td>
<td>3.5</td>
</tr>
<tr>
<td>3.0</td>
<td>0.7</td>
<td>0.9</td>
<td>1.7</td>
</tr>
<tr>
<td>4.0</td>
<td>3.6</td>
<td>3.4</td>
<td>3.5</td>
</tr>
<tr>
<td>5.0</td>
<td>1.2</td>
<td>1.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

All cells received 50 μg/ml cordycepin at 0 h. Virus-infected cells received 20 infectious units/cell FPV. Pulse labelling consisted of 5 μCi/ml [3H]-uridine (27.6 Ci/mmol) for 5 min. The cell sheets were prepared and counted as described in Tables 1 and 2.

(Penman, Rosbach & Penman, 1970). We have used autoradiography to see what effect the presence of 50 μg/ml of cordycepin had on the incorporation of [3H]-uridine into nuclear RNA in both uninfected and infected CEF.

Cordycepin had a differential effect on nuclear RNA synthesis in normal cells (Fig. 2). The incorporation of grains into nucleolar RNA was more rapidly and completely inhibited than into nucleoplasmic RNA and at 6 h after addition of the drug, residual RNA synthesis
**RNA synthesis in FPV-infected cells**

Table 4. *The effect of α-amanitin on total RNA synthesis in FPV-infected cells*  

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Radioactivity (ct/min)</th>
<th>% FPV control</th>
<th>% α-amanitin + FPV control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5138</td>
<td>80</td>
<td>22</td>
</tr>
<tr>
<td>0.5</td>
<td>3305</td>
<td>92</td>
<td>27</td>
</tr>
<tr>
<td>1.0</td>
<td>4310</td>
<td>99</td>
<td>54</td>
</tr>
<tr>
<td>1.5</td>
<td>5678</td>
<td>109</td>
<td>49</td>
</tr>
<tr>
<td>2.0</td>
<td>4801</td>
<td>64</td>
<td>49</td>
</tr>
<tr>
<td>3.0</td>
<td>5876</td>
<td>120</td>
<td>65</td>
</tr>
<tr>
<td>4.0</td>
<td>7709</td>
<td>112</td>
<td>132</td>
</tr>
</tbody>
</table>

*Primary cultures of CEF on Melinex strips were infected with FPV at a multiplicity of 20 p.f.u./cell. At various times before or after infection, duplicate infected or control cultures were treated with α-amanitin (20 μg/ml) for 1 h, followed by a 10 min pulse of [3H]-uridine. For comparison, infected and control cultures not treated with α-amanitin also received 10 min pulses of [3H]-uridine at the times shown above. After pulse labelling, the cells were washed with TCA, dried and immersed in toluene scintillator for detection of TCA insoluble radioactivity. Results listed above represent the average obtained from two separate experiments.*

was predominantly nucleoplasmic. RNA synthesis in the nucleoplasm of cells that received 50 μg/ml cordycepin at 0 h is shown in Table 3. The pattern of RNA synthesis determined for both nucleoplasm and nucleoli is shown in Fig. 3. Comparison of Table 3 with Table 2 indicates that although the absolute level of RNA synthesis was reduced by cordycepin, infection by influenza virus still induced two phases of nuclear RNA synthesis. The second peak, a proportion of which probably represents the synthesis of virus particle RNA (Scholtissek & Rott, 1970; Hastie & Mahy, 1973), was more pronounced. In these experiments, stimulation of RNA synthesis observed in influenza virus-infected cells occurred predominantly in the nucleoplasm; nucleolar labelling was less pronounced. These experiments suggest that the major site of influenza virus-induced RNA synthesis may be the cell nucleoplasm.

**The effects of α-amanitin**

α-amanitin inhibits the growth of fowl plague virus (Rott & Scholtissek, 1970), if added early in the growth cycle, and is also known to inhibit nucleoplasmic DNA-dependent RNA polymerase II (Kedinger et al. 1970). When added to uninfected CEF for 1 h it has little effect on overall RNA synthesis, but when present at the time of infection with FPV, there is a striking decrease in RNA synthesis (Mahy et al. 1972).

As virus growth is inhibited when α-amanitin is present in the medium continuously from the time of infection, we have examined the effects of the drug on RNA synthesis in control and FPV-infected cells by adding it at hourly intervals for 1 h, followed by a pulse of [3H]-uridine. This experiment was first performed by determining the levels of TCA insoluble radioactivity remaining in whole control or FPV-infected cells after addition of 20 μg/ml of α-amanitin for 1 h at hourly intervals, followed by a 10 min pulse of [3H]-uridine (5 μCi/ml, 27.6 Ci/mmol). The average results obtained from two separate experiments are shown in Table 4, and Fig. 4a. The pattern of RNA synthesis in both control and FPV-infected cells not treated with α-amanitin, but pulse labelled for 10 min with [3H]-uridine at hourly intervals is also shown in Table 4. A similar experiment was performed in which RNA synthesis was detected by autoradiography in control or FPV-infected cells, in the
Fig. 4. The effect of α-amanitin on RNA synthesis in FPV-infected CEF cells. Primary cultures of CEF on Melinex strips were infected with FPV at an input multiplicity of 20 p.f.u./cell. At various times duplicate infected and control cultures were treated with α-amanitin (20 μg/ml) for 1 h, followed by a pulse of [3H]-uridine (5 μCi/ml). (a) Cell preparations received [3H]-uridine for 10 min and were then washed with TCA, and immersed in toluene scintillator and counted. (b) Cells received [3H]-uridine for 5 min and were then fixed and processed for autoradiography. Results are expressed as percentage grains incorporated into α-amanitin treated FPV-infected cells and are compared with percentage grains incorporated into FPV-infected cells.

\[
\frac{\text{α-amanitin treated FPV-infected cells}}{\text{α-amanitin treated cells}}
\]

and are compared with percentage grains incorporated into

\[
\frac{\text{FPV-infected cells}}{\text{control cells}}
\]

○——○, α-amanitin treated cells; ●——●, control, untreated cells.

presence or absence of 20 μg/ml α-amanitin, and the results are shown in Fig. 4b. RNA synthesis in infected cells was significantly higher than in controls at 0.5 and 1 h (P < 0.0005) and at 3 h p.i. (P = 0.029), whereas in infected cells pre-treated for 1 h with α-amanitin, the RNA synthesis was significantly less than in uninfected α-amanitin treated cells at times up to 2 h post-infection (P < 0.0005 for each), but was significantly increased at 3 h (P = 0.02). The pattern of RNA synthesis determined by grain counts (Fig. 4b) corresponds
RNA synthesis in FPV-infected cells

Table 5. 'In situ' polymerase assay

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grain counts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>FPV-infected</td>
<td>5.89</td>
<td>4.96</td>
<td>8.69</td>
<td>10.84</td>
<td>6.42</td>
<td>4.88</td>
<td>5.99</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>4.67</td>
<td>3.59</td>
<td>2.68</td>
<td>4.14</td>
<td>4.12</td>
<td>2.36</td>
<td>2.77</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.51</td>
<td>0.04</td>
<td>&lt; 0.0005</td>
<td>&lt; 0.0005</td>
<td>&lt; 0.0005</td>
<td>&lt; 0.0005</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>FPV-infected</td>
<td>9.71</td>
<td>6.34</td>
<td>4.37</td>
<td>7.61</td>
<td>8.39</td>
<td>10.66</td>
<td>15.97</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>5.12</td>
<td>5.41</td>
<td>4.60</td>
<td>4.99</td>
<td>3.51</td>
<td>4.58</td>
<td>6.36</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>&lt; 0.0005</td>
<td>4.7</td>
<td>18.28</td>
<td>&lt; 0.0005</td>
<td>&lt; 0.0005</td>
<td>&lt; 0.0005</td>
<td>&lt; 0.0005</td>
</tr>
</tbody>
</table>

Nuclear and cytoplasmic grains were counted in 100 infected cells (input multiplicity 20 p.f.u./cell) and compared with uninfected cell counts. The probability (P) that differences arise by chance was determined using Student's t test, comparing the infected and control preparations at each given time. Actinomycin D was included in the polymerase assay mixture. The level of background grains averaged 4.6 and 1.97 grains per cell for cytoplasm and nuclei, respectively.

Fig. 5. Incorporation of [3H]-uridine triphosphate into the nuclei and cytoplasm of cells infected with FPV, fixed at various times after infection and incubated with an RNA-dependent RNA polymerase reaction mixture containing actinomycin D. Infected and control CEF cells were fixed at hourly intervals in cold alcohol-acetone. Cell strips were incubated with the reaction mixture at 32 °C for 45 min, fixed and washed in TCA, and prepared for autoradiography. Cytoplasmic and nuclear grains were counted for 100 cells at each time point. The background levels (4.60 grains/cell for cytoplasm, 1.97 grain/cell for nucleus) were deducted from mean values. ❏ — — ❏, nuclear grains; ▲ — — ▲, cytoplasmic grains.

almost exactly to that determined by scintillation counting (Fig. 4a); the autoradiographic data once again indicate that virus-induced stimulation of RNA synthesis occurs predominantly in the nucleoplasm. Treatment of FPV-infected cells with α-amanitin obliterates the first of the two distinguishable peaks of nucleoplasmic RNA synthesis.

The detection of RNA-dependent RNA polymerase 'in situ'

Many subsequent reports have confirmed and extended the observation of Ho & Walters (1966) that cells infected with influenza viruses contain large amounts of cytoplasmic RNA-dependent RNA polymerase associated with the microsomal element of fractionated
cells. More recently, it has been shown that this enzyme activity is associated with virus ribonucleoproteins in the cell cytoplasm (Skehel & Burke, 1969; Compans & Caliguiri, 1973). It is not clear whether this enzyme plays a role in virus replication. The experiments reported above fail to detect any significant level of virus-induced RNA synthesis in the cytoplasm of cells at any stage of infection. To demonstrate both the existence of and intracellular location of RNA-dependent RNA polymerase(s) we have used a modification of the in situ polymerase assay of Moore & Ringertz (1973). FPV-infected cells were fixed at hourly intervals following infection, and then incubated with a polymerase assay mixture.

Fig. 6. RNA synthesis in fixed CEF cells, detected by autoradiography. Control and FPV-infected cells were fixed at 6 h after infection, incubated with polymerase reaction mixture, and prepared for autoradiography. (a) Control cells; (b) FPV-infected cells.
RNA synthesis in FPV-infected cells

containing actinomycin D. After treatment with TCA, the cells were prepared for autoradiography, and the number and distribution of TCA insoluble grains was determined for nuclei and cytoplasm. The results are shown in Table 5 and Fig. 5 and the appearance of individual control cells and virus-infected cells at 6 h p.i. are shown in Fig. 6.

The nuclear grain count increased to a maximum at 3 h p.i., and then declined; the level of cytoplasmic activity was insignificant until 3 h p.i. and then increased to a maximum at 6 h p.i. The comparatively high levels of polymerase activity at the time of infection we attribute to the virus particle-associated transcriptase. Both the time of appearance and levels of activity of the nuclear and cytoplasmic RNA-dependent RNA polymerase(s) detected by autoradiography correspond closely to the values obtained with other techniques (Hastie & Mahy 1973).

DISCUSSION

The classical topographical studies of pox virus replication undertaken by Cairns (1960) first underlined the potential value of autoradiography as a technique for locating the site of virus-induced nucleic acid synthesis. Using this technique we could detect virus-induced, cytoplasmic RNA synthesis in cells that had been infected with the paramyxovirus, NDV, and exposed to short pulses of [3H]-uridine. Under similar conditions, we could not detect any cytoplasmic RNA synthesis in cells infected with an avian influenza A virus; increased levels of nucleoplasmic RNA synthesis were observed in these cells. Several experiments suggest that autoradiography is at least as sensitive as scintillation counting for the detection of RNA synthesis. The extent to which nucleoplasmic RNA synthesis is stimulated by FPV (Table 2) corresponds closely to the biphasic pattern of RNA synthesis observed in whole, infected cells (Borland & Mahy, 1968). It was found also (Fig. 4) that virtually identical results could be obtained when the effects of α-amanitin on virus-induced RNA synthesis were monitored either by autoradiography or scintillation counting. Allowing for the possibility that very low levels of cytoplasmic RNA synthesis might escape detection, we conclude that under the usual conditions of infection in intact cells, the principal site of influenza virus-induced RNA synthesis is the cell nucleus.

The main purpose of this paper was to detect the intracellular location of virus-induced RNA synthesis. We have no direct evidence that either peak of nucleoplasmic RNA synthesis represents the synthesis of virus RNA. However, RNA–RNA hybridization studies indicate that FPV RNA synthesis in chick fibroblasts begins at 1 h p.i. and reaches a peak at 3 h p.i. (Scholtissek & Rott, 1970), and in the same experimental system, RNA-dependent RNA polymerase activity increases in the cell nucleus rapidly from 1 h p.i., reaches a maximum at 3 to 4 h, then declines (Hastie & Mahy, 1973). We can detect maximal nuclear RNA-dependent RNA polymerase activity ‘in situ’ at 3 h p.i. (Fig. 5, Table 5). These findings suggest that at least a proportion of the virus-stimulated nucleoplasmic RNA synthesis found at 3 h is due to the synthesis of virus-specific RNA.

The significance of nucleoplasmic RNA synthesis in influenza virus replication is emphasized by studies with the inhibitors cordycepin and α-amanitin. We have shown (Fig. 2) that 50 μg/ml of cordycepin had an immediate effect on nucleolar RNA synthesis; it was suppressed more rapidly and to a greater extent than nucleoplasmic RNA, and furthermore, gross morphological alterations of nucleoli occurred within several hours. As the level of cordycepin used in this experiment does not suppress the yield of FPV (Mahy et al. 1973), we tentatively conclude that nucleoli are relatively unimportant in the replication of influenza viruses. Infection of cordycepin-treated cells with FPV also stimulates nucleoplasmic RNA synthesis (Fig. 3) in a manner similar to that observed in cells not treated with the
drug. In our experience, cordycepin is the only compound known to suppress cell RNA synthesis without inhibiting influenza virus growth. All inhibitors of DNA function, such as actinomycin D, also block virus production if added within 2 h post-infection. α-amanitin inhibits the nucleoplasmic DNA-dependent RNA polymerase of chick cells (Mahy et al. 1972), it inhibits the growth of FPV (Rott & Scholtissek, 1970), and it also inhibits the first peak of nucleoplasmic RNA synthesis induced by infection with FPV (Fig. 4). This latter finding suggests that cellular DNA transcription, mediated by polymerase II, accounts for the first peak of FPV-induced RNA synthesis and is essential for virus growth.

The possibility that there is virus-induced cytoplasmic RNA synthesis in FPV-infected cells arose from cell fractionation studies. An active, microsomal RNA-dependent RNA polymerase, capable of synthesizing RNA complementary to virus particle RNA has been detected in FPV-infected chick cells (Scholtissek & Rott, 1969), and this enzyme reaches a peak of activity at about 6 h post-infection (Mahy & Bromley, 1970). A similar enzyme has been found in the nucleus 3 h after infection (Hastie & Mahy, 1973). As we were unable to detect any active cytoplasmic RNA synthesis in whole cells (even when using the larger amounts of isotope and longer times in Table 1) we adopted a histochemical technique developed by Moore & Ringertz (1973). Fixed preparations of infected cells were incubated with a polymerase reaction mixture in the presence of actinomycin D, and then prepared for autoradiography. In this experiment, we detected cytoplasmic grains that increased in number to a maximum at 6 h. The pattern of grains found in the nuclei correspond closely in time to the appearance of the RNA-dependent RNA polymerase (Hastie & Mahy, 1973). Since the cytoplasmic RNA polymerase could be detected only under the artificial circumstances of this experiment, we conclude that it does not function in vivo. It might represent the accumulation of newly synthesized virus particle transcriptase molecules in infected cells. On the other hand, the nuclear enzyme detected by this technique may account for the second peak of nucleoplasmic RNA synthesis.

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REFERENCES


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