The Inhibition of HeLa Cell RNA Synthesis Following Infection with Vaccinia Virus

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SUMMARY

Vaccinia virus WR induces an immediate and rapid inhibition of HeLa S3 cell RNA synthesis as determined by pulse-labelling with [3H]uridine. The inhibition was independent of the purity of the infecting virus preparation and the multiplicity of infection over the range of 4 to 200 pk.f.u./cell. Inhibition was not evident in cells pre-treated with cycloheximide or following infection with u.v.- or heat-inactivated virus, suggesting that viral protein synthesis was required. There was no apparent selective inhibition of any particular species of RNA. Following infection, the uptake of [3H]uridine into cellular pools and the subsequent biosynthesis of UTP proceeded at the same rate as in mock-infected control cells. The rate of degradation of pre-labelled RNA was not enhanced in infected cells compared to controls. Analysis of the nuclear DNA-dependent RNA polymerase (EC 2.7.7.6) activities revealed a progressive and eventually total loss of RNA polymerase B activity, no obvious effect on RNA polymerase A and the presence of a viral RNA polymerase, the possible significance of which is discussed.

INTRODUCTION

The inhibition of host cell macromolecular synthesis is a common, but not invariable, consequence of virus infection. The inhibition of host cell protein synthesis has been shown to occur within 20 min of infection with vaccinia virus, at a time when viral protein synthesis is being initiated (Moss, 1968; Esteban & Metz, 1973). It is still a matter of some dispute whether this inhibition is mediated by a component of the infecting virus particle or a newly synthesized virus-induced protein (see Bablanian et al., 1978; Person & Beaud, 1978). In the case of host cell DNA synthesis, the accumulated evidence suggests that infection with vaccinia virus results in an early inhibition (Jungwirth & Launer, 1968) brought about by a virion-associated DNase activity. The DNase activity is specific for single-stranded regions of DNA which are known to occur around the replication fork and whose destruction would bring DNA replication to a halt (Olgiati et al., 1976; Pogo & Dales, 1973, 1974). In contrast, the inhibition of host cell RNA synthesis following poxvirus infection has received little attention. Becker & Joklik (1964) and Salzman et al. (1964) observed a 3 h lag prior to the onset of inhibition of HeLa cell RNA synthesis after vaccinia virus infection. However, Kit & Dubbs (1962) suggested that the inhibition occurs immediately after infection. The present study was undertaken to improve our understanding of the response of HeLa cell RNA synthesis to infection with vaccinia virus.

METHODS

Cells. HeLa S3 cells (Gibco-Biocult) were used throughout this work. They were propagated in 80 oz Winchester bottles as described by House & Wildy (1965) in Eagle's MEM supplemented with 10% newborn calf serum (Gibco-Biocult) and non-essential amino acids. The cells maintained in this way were used to provide suspension cultures for experimental work. The suspension cultures were prepared by seeding the cells at 1 x 10⁵ per ml in MEM (Joklik modified) supplemented with 5% newborn calf serum and non-essential amino acids. The cultures

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were agitated on an orbital shaker for volumes up to 500 ml and with a magnetic stirrer for larger volumes up to 6 l. The cells were used when they reached a concentration of $4 \times 10^6$ to $6 \times 10^5$ per ml.

**Virus.** Vaccinia virus strain WR was used throughout. Working stocks of the virus were prepared by infecting monolayer cultures of HeLa S3 cells in 80 oz Winchester bottles at an input multiplicity of 1 pk.f.u./cell in 20 ml MEM per bottle supplemented with 0-5% calf serum and 20 mM-MgCl$_2$. After adsorption of virus for 1 h a further 200 ml of MEM with 0-5% calf serum were added to each bottle and incubation was continued for 2 days by which time the cells showed an advanced c.p.e. The infected cells were shaken off the glass into the medium, harvested by centrifugation at 400 g for 20 min in the MSE Mistral 6L centrifuge and resuspended in phosphate-buffered saline (PBSA) at approximately $5 \times 10^7$ cell/ml. The cells were disrupted by ultrasonic vibration in a 'Megason' bath (Schuco International Ltd., London, U.K.) to release intracellular virus and the cell debris was removed by centrifugation at 1000 g for 10 min in an MSE bench centrifuge. The supernatant fluid was stored at $-20^\circ$C. The yield of virus obtained by this method was about 100 to 150 pk.f.u./cell.

These virus stocks were used to infect suspension cultures of HeLa S3 cells. Cells were harvested from suspension by centrifugation at 400 g for 20 min in the MSE Mistral 6L centrifuge and resuspended at $2 \times 10^7$ per ml in pre-warmed suspension medium containing 20 mM-MgCl$_2$. Unless otherwise stated, 40 pk.f.u./cell of vaccinia virus was added and the cells and virus inoculum incubated at 37 °C for 20 min with occasional gentle inversion to keep cells in suspension. After this adsorption period, cells were diluted to $5 \times 10^6$/ml in suspension medium containing 5% calf serum and incubated with either shaking or stirring. Points on the relevant graphs were timed from the addition of virus.

**Virus assay.** Titrations of infectivity were made by means of pock counts in groups of five 12-day-old chick embryos prepared for inoculation as described by McCarthy & Dumbell (1961).

**Virus inactivation.** Heat-inactivated vaccinia virus was prepared by heating 2 ml volumes of virus at 60 °C for 15 min.

The inactivation of vaccinia virus by u.v. irradiation was performed in 50 mm plastic Petri dishes at a distance of 15 cm from a Mineralight u.v. lamp (Ultraviolet Products Inc., San Gabriel, Cal., U.S.A.). The energy received was 0-8 J/m$^2$/s at a wavelength of 254 nm as determined by the ferrioxalate photoreduction method of Baxendale & Bridges (1955). Infectious virus could not be detected by egg inoculation with samples of undiluted material after either heat inactivation or u.v. irradiation.

**Cell fractionation.** Cells were removed from the suspension cultures, mixed with an equal volume of ice-cold isotonic buffer (150 mM-NaCl, 5 mM-EDTA, 50 mM-Tris-HCl pH 8-0 at 4 °C) and centrifuged at 200 g for 3 min. The cell pellet was washed three times in isotonic buffer and was finally resuspended at a concentration of $2 \times 10^7$ per ml in hypotonic buffer (50 mM-KCl, 5 mM-EDTA, 10 mM-Tris-HCl pH 8-0 at 4 °C). In experiments measuring RNA synthesis, the cells were allowed to swell for 5 min in the hypotonic buffer, and then disrupted by the addition of Triton X-100 to give a final concentration of 0-5% (v/v). For those experiments involving RNA polymerase, the cells were swollen in hypotonic buffer for 10 min and then ruptured in a homogenizer calibrated to produce maximum cell breakage with the minimum loss of nuclear material. The released nuclei were collected by centrifugation at 400 g for 2 min, washed three times in isotonic buffer and finally suspended at $5 \times 10^7$ per ml. In control experiments with uninfected cells labelled with [3H]thymidine, disruption resulted in more than 95% cell breakage and less than 4% of the whole cell radioactivity was found in the cytoplasmic fraction.

**Incorporation of [3H]uridine into RNA.** Samples of 10 ml ($5 \times 10^8$ cells) were removed from virus-infected and mock-infected cultures and pulse-labelled for 15 min with 0-2 µCi/ml [3H]uridine. After cell fractionation, duplicate 50 µl samples of nuclei were air-dried onto Whatman GF/C filters, washed three times in 5% TCA and once in 99% ethanol. The discs were dried and their radioactivity determined in an Intertechnique SL3000 scintillation spectrometer with an efficiency of counting of 35%. The scintillation fluid contained 0-4% (w/v) PPO and 0-005% (w/v) dimethyl POPOP in toluene. In experiments designed to measure the stability of RNA in vivo, cells were labelled with 0-5 µCi/ml [3H]uridine for 24 h prior to use. After washing three times in unlabelled medium, the cells were infected in the normal way. Their subsequent treatment is described in Results.

**Uptake and metabolism of [3H]uridine.** Samples of 120 ml ($6 \times 10^8$ cells) were withdrawn at various times after infection and pulse-labelled for 15 min with 0-2 µCi/ml [3H]uridine. Labelling was stopped by addition of an equal volume of cold isotonic buffer and the cells harvested and washed three times in 50 ml isotonic buffer. The final cell pellet was resuspended in 500 µl isotonic buffer and 20 µl amounts were dried onto four GF/C discs. Two discs were washed with 5% TCA to determine the acid-insoluble material, and the other two were counted without washing to determine the total count. The acid-soluble material was taken to be the difference between these two results.

The remainder of the cell suspension was used to follow the conversion of uridine to UTP by the method of Plagemann et al. (1969), except that 0-5 M-TCA was used for precipitation.

**RNA sedimentation.** Samples of 300 ml ($1 \times 10^9$ cells) were pulse-labelled with [3H]uridine (0-2 µCi/ml) for 15 min or 45 min at times 0, 4 and 10 h post-infection. Labelling was stopped by addition of an equal volume of cold isotonic buffer and the nuclei isolated by homogenization. RNA was extracted from the nuclei by the method of Scherrer & Darnell (1962). Ribosomal RNA markers were extracted from cells labelled for 24 h with [14C]uridine.
RNA synthesis inhibition by vaccinia virus

(0.025 μCi/ml) by the method of Bourguignon & Katz (1978). Samples of nuclear RNA were mixed with the rRNA markers and carefully layered on to linear 15 to 30% sucrose gradients containing 0.5% (w/v) SDS. Centrifugation was for 15 h at 66000 g in an SW50.1 rotor of the Beckman L2 ultracentrifuge. Each fraction was dried onto a GF/C disc and the TCA-insoluble radioactivity determined.

Solubilization, chromatography and assay of DNA-dependent RNA polymerases. Nuclei from infected or mock-infected cells were suspended in isotonic buffer at about 10^6 per ml. The RNA polymerases were solubilized and chromatographed on DEAE-Sephadex A-25 as described by Roeder & Rutter (1969).

The basic assay mix for the RNA polymerases was as follows: ATP, GTP, CTP, 0.6 mM; [3H]UTP, 0.5 mM (100 μCi/mol); Tris–HCl, 50 mM (pH 8.0 at 37 °C); 50 μl of nuclear suspension or solubilized RNA polymerase. The concentrations of divalent cations, (NH₄)₂SO₄ and α-amanitin used in the assays are detailed in the appropriate section of the results. All the RNA polymerase assays were performed in a final volume of 150 μl. The incubations were at 37 °C for 30 min and stopped by the addition of TCA to a final concentration of 5%. After 20 min at 4 °C, the precipitate was collected on GF/C filters, washed three times with 5% TCA and once with 99% ethanol, dried and counted. RNA polymerase activity is expressed in units, where 1 unit is equivalent to the incorporation of 1 pmol UMP into TCA-insoluble material in 30 min at 37 °C.

Determination of molecular weight. The molecular weights of the RNA polymerases were determined by both gel filtration and glycerol gradient centrifugation. For gel filtration, Bio-Gel A-15 (Bio-Rad), with a molecular weight fractionation range of 1.0 x 10^6 to 1.5 x 10^6, was used and calibrated with aldolase, catalase, ferritin and thyroglobulin (high molecular weight calibration kit, Boehringer). With ferritin as a marker, the molecular weights were also determined on 15 to 35% glycerol gradients using the formula of Martin & Ames (1961).

Other assays. For the estimation of protein concentrations the Bennet (1967) modification of the assay of Lowry et al. (1951) was used. The DNA concentration of nuclei preparations was determined by the Giles & Myers (1965) modification of the Burton (1955) assay.

Materials. Uridine, UMP, UDP, UDP-glucose, actinomycin D, cycloheximide, sucrose (grade I, RNase-free), ammonium sulphate (grade I), DNA (type I, from calf thymus), 2-mercaptoethanol and Trizma base (reagent grade) were purchased from Sigma. ATP, CTP, GTP, α-amanitin were bought from Boehringer. L-[U-14C]Valine (275 mCi/mmol), L-[U-14C]phenylalanine (513 mCi/mmol), [5,6-3H]uridine (42 C/mmol), [2-14C]uridine (50 mCi/mmol), [5-3H]uridine-5'-triphosphate, ammonium salt (10.9 Ci/mmol) were from Amersham. Thioglycerol, PPO and dimethyl PPO were from Koch-Light Laboratories. All other chemicals were of AnalR grade and were purchased from BDH. DEAE-Sephadex A-25 was from Pharmacia.

RESULTS

Inhibition of RNA synthesis by vaccinia virus

The inhibition of RNA synthesis in vaccinia virus-infected cells compared to mock-infected controls is shown in Fig. 1. The rate of RNA synthesis in uninfected cells remained relatively constant throughout the time of the experiment whereas there was an immediate and rapid reduction in the rate of RNA synthesis in vaccinia virus-infected cells, the extent of this inhibition reaching 60% by 3 h after infection and approximately 90% by 9 h after infection. Varying the multiplicity of infection (4, 40 and 200 pk.f.u./cell) did not significantly affect the rate and extent of the inhibition being detected. This same pattern of inhibition was observed when the cells were fractionated by Dounce homogenization rather than by detergent, or infected with purified vaccinia virus (results not shown). In all subsequent experiments, therefore, unpurified virus was used.

Requirements for inhibition of RNA synthesis

Live virus was essential for the inhibition. Heat-inactivated or u.v.-inactivated vaccinia virus was unable to inhibit host cell RNA synthesis (Fig. 1). After heat inactivation, neither infectivity nor virion-associated DNA-dependent RNA polymerase activity could be detected. After u.v. inactivation, no infectivity and about 1% RNA polymerase activity could be detected. As heat affects the virion proteins and u.v. primarily affects the viral DNA, the most obvious link between the two is the DNA-dependent RNA polymerase associated with the virion, i.e. heat inactivates the enzyme directly and u.v. blocks its activity by severely damaging its template. The result is an inability to synthesize virus-specific mRNA with a concomitant loss of ability to synthesize virus-specific proteins. The requirement for de novo protein synthesis was therefore investigated using cycloheximide. Pilot experiments showed that within 15 min of addition of 50 μg/ml cycloheximide, protein synthesis could not be detected but there was also an
Fig. 1. Effect of different multiplicities of infection and inactivated virus on the inhibition of nuclear RNA synthesis. O, Mock-infected cells; □, 4 pk.f.u./cell; ●, 40 pk.f.u./cell; Δ, 200 pk.f.u./cell; ▲, u.v.-inactivated virus; ■, heat-inactivated virus.

Fig. 2. Effect of cycloheximide (50 μg/ml) on vaccinia virus-induced inhibition of HeLa cell nuclear RNA synthesis. RNA synthesis (3H incorporation): O, mock-infected cells; ●, virus-infected cells; □, mock-infected cells and cycloheximide; ■, virus-infected cells and cycloheximide. Protein synthesis (14C incorporation): ▲, virus-infected cells; Δ, virus-infected cells and cycloheximide. Protein synthesis in mock-infected cells with and without cycloheximide was not significantly different from that shown for virus-infected cells and has therefore been omitted for clarity.

Table 1. Effect of vaccinia virus infection on the uptake of uridine and its conversion to UTP*

<table>
<thead>
<tr>
<th>Time after infection (h)</th>
<th>Treatment</th>
<th>C.p.m. in acid-soluble pool per 10⁷ cells</th>
<th>Percentage of total c.p.m. recovered from the chromatogram</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uridine UMP UDP UDP-G UTP</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Mock</td>
<td>27277 (±1161) 27.3 (±2.1) 14.7 (±2.1) 41.9 (±4.3)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Mock</td>
<td>25064 (±2018) 14.5 (±1.4) 27.2 (±2.7) 13.0 (±3.1) 45.2 (±5.5)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Mock</td>
<td>27164 (±1700) 15.6 (±0.4) 28.7 (±1.5) 10.5 (±1.0) 45.2 (±2.1)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Infected</td>
<td>26386 (±2904) 15.8 (±0.4) 30.7 (±2.5) 13.3 (±1.4) 40.1 (±3.4)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Infected</td>
<td>24627 (±1866) 15.0 (±0.7) 22.3 (±5.8) 19.1 (±3.9) 43.6 (±2.6)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Infected</td>
<td>25530 (±659) 15.3 (±0.7) 28.4 (±2.9) 12.8 (±1.7) 43.5 (±3.7)</td>
<td></td>
</tr>
</tbody>
</table>

* The results and standard errors of the means are taken from three repeat experiments.

approximately 50% inhibition of RNA synthesis even in the absence of virus. The effect of this concentration of cycloheximide upon vaccinia virus-induced inhibition of cellular RNA synthesis is shown in Fig. 2. The drug was added to cell cultures 30 min prior to infection and the cells were pulse-labelled with [3H]uridine and continuously labelled with 0.025 μCi/ml [14C]phenylalanine and 0.025 μCi/ml [14C]valine. There was a 40 to 50% inhibition of RNA synthesis by cycloheximide as compared with control cells, but this reduced rate of RNA synthesis was maintained at a consistent level throughout the duration of the experiment. Similarly, RNA synthesis within infected cycloheximide-treated cells was also maintained at about 50% of that in control uninfected cells. Thus, vaccinia virus did not inhibit host cell RNA synthesis in the absence of protein synthesis.

Effect of vaccinia virus infection on the uptake and metabolism of uridine

Viral interference with the uptake and/or metabolism of RNA precursors are two possible mechanisms by which RNA synthesis could be inhibited. These possibilities were examined and the results are shown in Table 1. The uptake of [3H]uridine into acid-soluble intracellular pools
RNA synthesis inhibition by vaccinia virus

Fig. 3. Degradation of pre-labelled nuclear RNA in mock-infected (○) and virus-infected (●) cells. Following infection, the cells were resuspended and maintained in (a) unsupplemented suspension medium, (b) suspension medium containing 5 mM unlabelled uridine, or (c) unsupplemented medium to which 10 µg/ml actinomycin D was added 1 h after infection (arrow).

after a 15 min pulse was not significantly different between infected and mock-infected cells even at 10 h after infection. Thus, it is clear that the inhibition of RNA synthesis was not the result of an impaired uptake of uridine. Chromatography failed to detect any labelled material within the region occupied by uridine, suggesting a rapid conversion to UMP. It can be seen from Table 1 that the percentage of counts in UMP, UDP and UTP were not significantly different in mock-infected or vaccinia virus-infected cells at various times after infection. This also applied to UDP-glucose (UDP-G), showing that acid-soluble uridine metabolites were not being channelled at an increased rate into reactions other than UTP synthesis. Labelled material was not detected in any other part of the chromatogram.

Effect of vaccinia virus infection on pre-formed RNA

A large increase in the rate of degradation of RNA after virus infection could result in an apparent inhibition of RNA synthesis. Accordingly, cells which had been pre-labelled for 24 h with [3H]uridine were infected or mock-infected and the loss of acid-insoluble radioactivity in the nuclei was determined at various times after infection. The results are shown in Fig. 3. In Fig. 3(a) the rate of degradation appeared slightly greater in the mock-infected cells but these curves represent a balance between degradation and re-utilization of the released acid-soluble uridine. The inhibition of RNA synthesis in virus-infected cells would suggest that this re-utilization would be minimal but in an attempt to reduce it even further, the experiment was repeated in the presence of 5 mM unlabelled uridine. In this case, there was no significant difference in the rate of degradation of RNA in infected and mock-infected cells. These experiments were repeated in the presence of 10 µg/ml actinomycin D added 1 h after infection to permit some of the virus-induced inhibitor to be synthesized. Under these conditions, re-utilization of label is prevented completely and again there was no significant difference between infected and mock-infected cells (Fig. 3c). These results indicate that the inhibition of host cell RNA synthesis was real and not the result of a large increase in the rate of RNA degradation.

Selectivity of inhibition of RNA synthesis

The results presented so far have demonstrated the inhibition of total RNA synthesis in nuclei but do not permit conclusions to be drawn about the selectivity of the inhibition. This aspect of the inhibition was examined by centrifuging the RNA extracted after pulse-labelling at different times after infection with [3H]uridine on 15 to 30% sucrose gradients. The sedimentation profiles after a 15 min pulse (Fig. 4a) and a 45 min pulse (Fig. 4b) showed no obvious selectivity in the inhibition of synthesis of different RNA species.
Fig. 4. Sedimentation of HeLa cell nuclear RNA synthesized during (a) a 15 min pulse and (b) a 45 min pulse. ○, Cells 0 h after infection; ●, cells 4 h after infection; △, cells 10 h after infection. The sedimentation of RNA extracted from mock-infected cells at these times was not significantly different from that of RNA extracted immediately after addition of virus. These profiles have therefore been omitted for clarity.

Effect of vaccinia virus infection on HeLa cell RNA polymerases

The different nuclear RNA polymerases can be assayed independently by varying the ionic strength in combination with the use of the fungal toxin, α-amanitin. Fig. 5(a) shows the changes in RNA polymerase B activity in nuclei from control and virus-infected cells over a period of 20 h. The activity in nuclei from mock-infected cells increased slowly during this period but the activity of RNA polymerase B in nuclei from virus-infected cells was inhibited within the first 2 h post-infection and continued to decline until 10 to 12 h, whereupon the activity was completely inhibited. Measurements of RNA synthesis by pulse-labelling, performed in parallel with the polymerase studies (results not shown), disclosed a close relationship between the rates of inhibition of each. RNA synthesis was inhibited 50% by 3 h post-infection, 90% by 7 h and 100% by 11 h; by comparison, the inhibition of RNA polymerase B activity at these times was 42%, 80% and 96%.

Fig. 5(b) shows the changes of endogenous nuclear RNA polymerase A activity in control and virus-infected cells during the same 20 h. Throughout this period the RNA polymerase A activity of mock-infected cells remained virtually constant. The response following vaccinia virus infection was totally unpredicted. Over the first 2 h post-infection the activity resembled that in mock-infected controls, but between 2 and 4 h a stimulation of activity was observed
RNA synthesis inhibition by vaccinia virus

Fig. 5. DNA-dependent RNA polymerase activity in nuclei from infected (●) and mock-infected (○) cells. (a) RNA polymerase B; the standard assay contained 100 mM-(NH₄)₂SO₄ and 1.75 mM-MnCl₂. The activity represents the difference when assayed in the presence and absence of 5 μg/ml α-amanitin. (b) RNA polymerase A; the standard assay contained 12.5 mM-(NH₄)₂SO₄, 4 mM-MgCl₂ and 5 μg/ml α-amanitin.

which continued until reaching a maximum between 10 and 12 h after infection. From 12 h there was a steady decline in the nuclear polymerase A activity and by 20 h it had returned to the levels of mock-infected controls. The RNA polymerase activities were solubilized from nuclei isolated 10 h after vaccinia virus or mock-infection of HeLa S3 cells. The activities were subjected to DEAE-Sephadex chromatography and the resulting profiles are shown in Fig. 6. The elution pattern from nuclei of mock-infected cells showed the expected result (Fig. 6a), with RNA polymerase A eluting at 0.17 M-(NH₄)₂SO₄ and RNA polymerase B eluting at 0.27 M-(NH₄)₂SO₄. This elution pattern was dramatically altered when the RNA polymerases from infected nuclei were run (Fig. 6b). In this case, RNA polymerase A was present in normal amounts, a new and large peak of activity appeared in the 0.1 M-(NH₄)₂SO₄ wash and, as would be expected from the results with intact nuclei, RNA polymerase B was completely absent.

Properties of the solubilized RNA polymerases

A variety of biochemical properties of RNA polymerases A and B were compared to those of the new RNA polymerase activity eluting at 0.1 M salt. The results are summarized in Table 2. The properties shown for RNA polymerases A and B are those which would be expected from uninfected HeLa cells. The other RNA polymerase differs from both the normal cellular activities in a number of respects. Compared to RNA polymerase A, it had a slightly higher ammonium sulphate optimum of 20 mM, a higher Mn²⁺/Mg²⁺ activity ratio and a significantly higher molecular weight. It differed from RNA polymerase B in having a lower ammonium sulphate optimum and in being insensitive to α-amanitin. It can also be seen from Table 2 that the new RNA polymerase activity was inactivated by anti-vaccinia virus serum whereas the host enzymes were not.

DISCUSSION

Infection of suspension cultures of HeLa S3 cells with vaccinia virus is followed by an immediate and rapid inhibition of host cell RNA synthesis. These results are consistent with those of Kit & Dubbs (1962) but we have been unable to detect a lag phase period for this inhibition as described by Becker & Joklik (1964) and Salzman et al. (1964). The reason for this discrepancy is unclear. It is not due to the degree of purity of the virus preparation, the
multiplicity of infection or the method of cell disruption used to prepare nuclei, none of which has a significant effect on the kinetics of the inhibition. This early inhibition is consistent with the vaccinia virus-induced inhibition of protein synthesis (Moss, 1968) and DNA synthesis (Jungwirth & Launer, 1968) as well as with the early inhibition of RNA synthesis observed after infection with other DNA viruses, e.g. herpes simplex virus (Flanagan, 1967; Roizman et al., 1965) and frog virus 3 (Maes & Granoff, 1967).

Several lines of evidence indicate that the inhibition of host cell RNA synthesis required vaccinia virus-induced protein synthesis and was not a result of a component of the infecting virus particles. Firstly, a multiplicity of infection between 4 and 200 pk.f.u./cell had little effect on the kinetics of the inhibition. Live virus was required, and inactivation by heat or u.v. light prevented the inhibition which suggests that both viral protein and nucleic acid were necessary. The most likely candidate for this requirement is the virion-associated DNA-dependent RNA
RNA synthesis inhibition by vaccinia virus

Table 2. Comparison of the properties of solubilized RNA polymerases from mock-infected and vaccinia virus-infected HeLa cell nuclei

<table>
<thead>
<tr>
<th>Property</th>
<th>RNA polymerase A</th>
<th>RNA polymerase B</th>
<th>0.1 M-RNA polymerase</th>
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<tr>
<td>(NH₄)₂SO₄ optimum</td>
<td>12.5 mM</td>
<td>90 mM</td>
<td>20 mM</td>
</tr>
<tr>
<td>pH optimum</td>
<td>7.8</td>
<td>8.2</td>
<td>8.0</td>
</tr>
<tr>
<td>Mn²⁺/Mg²⁺ activity</td>
<td>1:1</td>
<td>4:9</td>
<td>4:8</td>
</tr>
<tr>
<td>α-Amanitin (5 µg/ml)</td>
<td>Insensitive</td>
<td>Sensitive</td>
<td>Insensitive</td>
</tr>
<tr>
<td>Inhibition by anti-vaccinia serum cf. pre-immune (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µl</td>
<td>0</td>
<td>13</td>
<td>48</td>
</tr>
<tr>
<td>20 µl</td>
<td>0</td>
<td>12</td>
<td>55</td>
</tr>
<tr>
<td>Mol. wt.</td>
<td>479000</td>
<td>531000</td>
<td>549500</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>440000</td>
<td>507600</td>
<td>507600</td>
</tr>
</tbody>
</table>

polymerase which is essential for the synthesis of mRNA. Finally, cycloheximide prevents the inhibition of RNA synthesis. The drug alone inhibits RNA synthesis by 50% which makes interpretation difficult, but it is clear that in the absence of protein synthesis, the virus-induced inhibition of RNA synthesis is abolished.

The differential inhibition of different species of nuclear RNA has been observed in herpesvirus- (Wagner & Roizman, 1969) and picornavirus- (Bienz et al., 1978) infected cells. We have not found any such selectivity in the case of vaccinia virus-infected cells. Sedimentation analysis of RNA extracted at different times after infection after a 15 min pulse indicated that all species of RNA were inhibited. The possibility that a 15 min pulse was insufficient for the synthesis of all classes of cellular RNA (Soeiro et al., 1968) and, in particular, for species of RNA that may be transcribed at reduced rates following infection, prompted the use of a pulse-labelling period extended to 45 min. Again, sedimentation analysis did not reveal any selective inhibition. These results are in agreement with those of Becker & Joklik (1964) and Salzman et al. (1964) who observed a similar suppression of synthesis of all species of RNA once the inhibition had been initiated.

Several mechanisms for the inhibition of host cell RNA synthesis have been explored. Firstly, the uptake of [³H]uridine into intracellular pools was measured by pulse-labelling of infected and uninfected cells. No differences were observed that would indicate that a reduction of uridine transport caused the inhibition of RNA synthesis. Similar results have been found with other virus-cell systems including vesicular stomatitis virus (Weck & Wagner, 1978; Wu & Lucas-Lenard, 1980), herpesviruses (Racusanova et al., 1972) and picornaviruses (Martin et al., 1961).

A second possible mechanism of RNA synthesis inhibition is a block in the pathway from uridine to UTP, particularly in view of the reduction in uridine kinase activity reported by Kit (1964) after vaccinia virus infection. However, the data in Table 1 clearly demonstrate that this is not the mechanism employed to inhibit RNA synthesis. Neither of these results is unexpected, as a large reduction in uridine uptake or UTP synthesis would also have the effect of inhibiting viral mRNA synthesis.

A third possible mechanism of RNA synthesis inhibition explored in this work is an increased rate of degradation of the RNA product. Thus, inhibition would be apparent if the RNA products were degraded at a rate which equalled or exceeded the rate of synthesis. A role for a vaccinia virus nuclease in the inhibition of cellular DNA synthesis has already been postulated (Olgiati et al., 1976). Similarly, it may be suggested that a virus-induced RNase activity degrades the newly synthesized host cell RNA. Accurate determinations of the rates of RNA degradation depend upon the re-utilization of solubilized nucleotides being suppressed by the addition of inhibitors of RNA synthesis, usually actinomycin D. The application of this technique to the present study is limited by the efficiency with which the actinomycin D also suppresses the synthesis of viral mRNA, a necessary prerequisite for cellular RNA synthesis inhibition. Nevertheless, despite these difficulties the results in Fig. 3 strongly suggest that an increased rate of
RNA degradation is not the mechanism used by vaccinia virus to produce an apparent reduction in cellular RNA synthesis.

The inhibition of host cell RNA synthesis can be partly explained by the loss of RNA polymerase B activity in infected cell nuclei. This is not the complete explanation, however, because RNA polymerase A appears to retain its normal level of activity, at least after solubilization. Because of the masking effect of the high level of the RNA polymerase eluted at 0.1 M salt, it is not possible to show whether RNA polymerase A is active in nuclei. Matsui et al. (1976a) have shown that RNA polymerase A elutes as a single peak from DEAE-Sephadex but can be separated into two subtypes, Aa and Ab, on phosphocellulose. The evidence suggests that the Aa enzyme is transcriptionally inactive \textit{in vivo} (Matsui et al., 1976b). It is possible, therefore, that following vaccinia virus infection, all polymerase A molecules are converted to the Aa subtype such that the enzyme becomes inactive \textit{in vivo} though exhibiting normal levels of activity after solubilization.

A stimulation of an \textalpha;-amanitin-insensitive nuclear RNA polymerase activity in BHK21/C13 cells infected with the IHD strain of vaccinia virus was observed by Constanzo et al. (1970). They attributed this activity to the contamination of their nuclear preparations with viral polymerase from the cytoplasm. Such an interpretation would explain our conflicting observations of inhibition of nuclear RNA synthesis and enhanced nuclear RNA polymerase activity. Certainly our evidence strongly suggests a viral origin for the new polymerase, i.e. its biochemical properties closely resemble the solubilized viral enzyme described by Baroudy & Moss (1980) and its neutralization by vaccinia virus-specific antiserum. However, repeated washings of the nuclei in buffers containing Triton X-100 and pre-incubation of the nuclei in the presence of Triton X-100 had no effect upon the activity of the nuclear RNA polymerase. This raises the possibility that some of the viral enzyme is transported to the nucleus, in a form which does not require activation by Triton X-100, providing a more defined role for the host nucleus which several workers have implicated in the replication of poxviruses (Pennington & Follett, 1974; Gafford & Randall, 1976; Bolden et al., 1979; Hruby et al., 1979a, b; Silver et al., 1979). Since this paper was submitted, Wing & Weissbach (1984) have published evidence for the nuclear involvement of vaccinia virus RNA polymerase.

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\textbf{REFERENCES}


RNA synthesis inhibition by vaccinia virus


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