Patterns of Transcription of Messengers Containing Poly A in Vaccinia Virus-infected Cells

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SUMMARY

Messenger RNA (mRNA) with sequences of polyriboadenylic acid (poly A) has been isolated from the cytoplasm of vaccinia virus-infected cells after a short pulse label with radioactive uridine. No labelled RNA containing poly A was isolated from mock-infected cells under the same conditions. mRNA synthesized at various times during the replicative cycle was examined by polyacrylamide gel electrophoresis and compared with the mRNA synthesized in the presence of inhibitors of protein and DNA synthesis. No difference was detected between ‘class 1’ and ‘class 2 early’ mRNA species although ‘late’ RNA species were on average considerably larger than the ‘early’ species.

INTRODUCTION

The replicative cycle of vaccinia virus can be separated clearly into ‘early’ and ‘late’ stages (Joklik et al. 1967). In the ‘early’ stage, before DNA replication, a number of enzymes are induced, their synthesis being switched off in the later stages of the infection as DNA synthesis proceeds (McAuslan, 1963). It has been suggested that the early functions expressed by the parental genome can be subdivided further into two classes (Kates & McAuslan, 1967). Upon infection the outer coat of the virus particle is removed, the virus core is released into the cytoplasm of the cell (Dales, 1963) and the particle-associated DNA-dependent RNA polymerase then transcribes a portion of the parental genome. An initial burst of mRNA synthesis (‘class 1 early’ mRNA) then occurs, the virus core is broken down to release the parental genome (second stage uncoating) and a further burst of mRNA synthesis occurs (‘class 2 early’).

Several groups of workers (Salzman, Shatkin & Sebring, 1964; Oda & Joklik, 1967; Kates, 1970) have used sucrose velocity gradient sedimentation to compare the nature of the mRNA synthesized in vaccinia-infected cells at ‘early’ and ‘late’ times in the infection. In all cases an overall increase in size of the vaccinia-specified messengers was observed at late times in the infection but the estimates of the size ranges of the RNA species were very unsatisfactory, as the RNA species always sedimented as heterogeneous zones in the sucrose gradients.

These observations, coupled with the recent finding of Kates (1970) that vaccinia-specified mRNA synthesized both in vivo and in vitro has poly A sequences attached, stimulated us to re-examine by a high-resolution method the vaccinia mRNA populations present at ‘early’ and ‘late’ times. We have studied particularly the mRNA species containing poly A and have characterized them by polyacrylamide gel electrophoresis.
METHODS

Media. Eagle’s medium (Vantsis & Wildy, 1962) was supplemented with either 10% tryptose phosphate broth and 10% rabbit serum (ETR), 10% rabbit serum (ER10), 2% rabbit serum (ER2) or 10% rabbit serum with the final medium in 0.02 M-MgCl2 (ER10Mg).

Virus and cells. The Lister strain of vaccinia virus was used in all experiments. Rabbit kidney cells (RK 13) (Beale, Cristofinisi & Furminger, 1963) were grown in ETR as described by House & Wildy (1965).

Pulse labelling of mRNA. Confluent monolayers of RK 13 cells in 50 mm plastic Petri dishes were infected at an input multiplicity of 15 to 20 p.f.u./cell with vaccinia virus in 0.2 ml ER10Mg. This multiplicity was sufficient to give good one-step growth conditions. After adsorption for 1 h at 37°C the excess inoculum was removed and replaced with 5 ml ER10. At various times after infection the cells were pulse labelled for 20 min with [3H]- or [14C]-uridine (The Radiochemical Centre, Amersham) contained in 2 ml ER2 per dish. The labelled uridine was added at a concentration of 12.5 to 25 μCi/ml if the RNA was to be analysed by polyacrylamide electrophoresis, or at 125 to 250 μCi/ml if the RNA was to be used for molecular hybridization. Normally 105 cells and 2 dishes were used for each preparation.

Extraction of RNA. Pulse labelled cells were harvested, washed with 0.15 M-NaCl, 4 mM-phosphate, pH 7.2 (PBS), resuspended in 0.14 M-NaCl, 1.5 mM-MgCl2, 0.01 M-tris, pH 8.5 (1 x 106 cells/ml) and extracted with phenol at 0°C in the presence of 1% Tween 40 and 0.5% deoxycholate (Atherton & Darby, 1974). The phenol phase was re-extracted with 0.1 M-tris buffer solution, pH 9 (Brawerman, Mendecki & Lee, 1972) and the aqueous phases were combined and re-extracted with phenol at 0°C. The RNA was precipitated by the addition of 2 vol. of ethanol. [32P]-Ribosomal RNA from RK 13 cells was added routinely to the cells prior to phenol extraction and a sample of the RNA was analysed by polyacrylamide gel electrophoresis after extraction to check for ribonuclease degradation.

Preparation of mRNA containing poly A. The extracted RNA was redissolved in 1 ml 0.5 M-KCl, 1 mM-MgCl2, 0.01 M-tris, pH 7.6 and the mRNA species containing poly A were bound to nitrocellulose membrane filters as described by Brawerman et al. (1972). The filters were washed with the buffer solution and the bound RNA was eluted with 1 ml 0.3% SDS in 0.1 M-tris, pH 9. The RNA was precipitated with 2 vol. of ethanol in the presence of 80 μg of yeast carrier RNA. [32P]-Ribosomal RNA from RK 13 cells was added to the elution buffer solution. In double-label experiments with [3H]- and [14C]-labelled mRNA, the carrier used was 80 μg RK 13 rRNA. This also acted as a marker in the subsequent polyacrylamide electrophoresis.

Metabolic inhibitors. Cycloheximide was used to inhibit protein synthesis and cytosine arabinoside to inhibit DNA synthesis. Both inhibitors were used at a concentration of 25 μg/ml. The cells were pre-incubated with the inhibitors for 1 h before infection and they were then present in the system until the cells were harvested. Cycloheximide at 25 μg/ml was shown to inhibit incorporation of [3H]-lysine into trichloroacetic acid (TCA)-precipitable material by more than 97% and reduced to 24 h yield of virus by more than 99%. Similarly, cytosine arabinoside at 25 μg/ml inhibited incorporation of [3H]-thymidine in TCA-precipitable material to less than 1% and reduced the 24 h yield of infectious virus to less than 2%.

Polyacrylamide gel electrophoresis. RNA samples were analysed on polyacrylamide gels containing 0.5% agarose by the procedure of Peacock & Dingman (1968). The samples
were applied to the gels in 25 µl of the electrophoresis buffer solution containing 10 % (w/v) sucrose and 0.2 % SDS. At the completion of the runs, marker RNA species were made visible by immersing the gels in 5 % TCA at 0 °C for several min. Radioactivity profiles were obtained by slicing the gels, leaching the slices by soaking overnight at room temperature in Soluene-100 (Packard) and measuring the activity by liquid scintillation using a toluene based scintillator.

**Sucrose gradient sedimentation.** RNA samples were analysed by sedimentation through linear 5 to 20 % (w/v) sucrose gradients in 0.1 M-NaCl, 5 mM-EDTA, 0.05 M-tris, pH 7.4. The samples were sedimented for 5 h at 0 °C and 120000 g in the SW 40 MSE rotor. [3P]-labelled *Escherichia coli* rRNA was used as a marker in the gradients.

**Molecular hybridization.** The molecular hybridization technique of Gillespie & Spiegelman (1965) was used with the following modifications: DNA (50 µg) in 0.01 × SSC (SSC = 0.15 M-NaCl, 0.015 M-sodium citrate) was denatured at 0 °C at pH 12.8 and the solution was neutralized. Denatured DNA (5 µg) was immobilized on each membrane filter (Sartorius, 34 Göttingen, W. Germany) by slow filtration of 5 ml of DNA solution in 2 × SSC. Hybridization was at 66 °C for 75 h with the labelled RNA in 2.5 ml 2 × SSC. Excess RNA was removed by washing each side of the filters with 2 × SSC from a pipette, immersing in three consecutive 250 ml vol. of 2 × SSC and finally washing both sides under vacuum with 5 × 15 ml samples of 2 × SSC. RNA bound to the filters but not hybridized to DNA was removed by incubating the filters for 30 min at 37 °C in 2 ml 2 × SSC containing 100 µg RNase-A and 100 units RNase T1 (pre-heated at 85 °C for 10 min). The filters were then washed again as described above and the radioactivity bound measured by liquid scintillation counting.

**Extraction of DNA.** Vaccinia virus (approx. 10^{11} p.f.u.) was purified by sedimenting through 35 % (w/v) sucrose at 15 000 g for 90 min. The pellet was treated with 50 µg/ml electrophoretically pure DNase at 37 °C for 90 min (Worthington Biochemical Corp.) and then sedimented once more through sucrose. The virus DNA was extracted by the method of Joklik (1962) with modifications suggested by Oda & Joklik (1967). Pre-labelling of the host DNA with [3H]-thymidine indicated less than 0.2 % contamination by weight of the virus DNA with host material.

DNA was extracted from RK 13 cells by first preparing the nuclei (Borun, Scharff & Robbins, 1967), lysing the nuclei by treatment with 0.5 M-NaCl, 50 mM-MgCl_{2}, 0.01 M-tris, pH 7.4 and finally extracting with chloroform/amyl followed by phenol/0.1 % SDS. The DNA was incubated with 50 µg/ml RNase-A and 20 units RNase T1 (heated at 85 °C for 10 min before use) and then extracted several times with cold phenol to remove the RNase.

**RESULTS**

**Course of mRNA synthesis**

Infected cells were exposed to pulses of [3H]-uridine at various times after infection and the synthesized mRNA species containing poly A were extracted and analysed by electrophoresis in 2.4 % polyacrylamide gels. The results are shown in Fig. 1. At 1.5 h after infection the mol. wt. of the RNA in the peak fractions on the gel was approx. 0.5 × 10^6. By 3 h the pattern had altered dramatically and the RNA in the peak fractions was approx. 1.3 × 10^6. Little change was observed at later times. It therefore appeared that in this system the ‘late’ mRNA synthesis had begun by 3 h after infection.

A number of essential controls were performed to confirm that the patterns observed represented undegraded virus-specified messengers.
Fig. 1. Analysis of RNA containing poly A extracted from the cytoplasm of vaccinia-infected cells at (a) 1.5 h, (b) 3.0 h, (c) 4.5 h and (d) 6.0 h after infection. Infected cells were pulse labelled for 20 min with 50 μCi [3H]-uridine, the RNA extracted and analysed in 2.4 % polyacrylamide gels run for 2 h at 5 mA per gel. The positions of [32P]-labelled RK 13 rRNA markers are shown.

**Nuclease degradation**

In order to determine whether RNA was degraded during the extraction procedure, [32P]-rRNA from RK 13 cells was added to the cells at the time of harvesting. This RNA was extracted along with the total cytoplasmic RNA. As the rRNA did not bind to the nitrocellulose membrane filters used to isolate the RNA species containing poly A, it was analysed by polyacrylamide gel electrophoresis prior to passage through the filter. A typical rRNA profile was obtained and no degradation was detected. In addition, [32P]-rRNA was added to the buffer used to elute the poly A messengers which were bound to the filters. This RNA also remained intact during the elution. It was therefore likely that the mRNA species containing poly A which were examined by polyacrylamide gel electrophoresis were undegraded.

Molecular hybridization experiments were also done as a further control to demonstrate that the RNA synthesized during the short pulses was virus specified (see ‘Molecular hybridization’).

**Mock-infected cells**

Mock-infected cells were labelled for 20 min with [3H]-uridine and the cytoplasmic mRNA species containing poly A were extracted. The RNA was analysed on a 2.4 % polyacrylamide gel and from the profile obtained it was apparent that the level of host mRNA which was synthesized during the pulse and transported to the cytoplasm was very low and that this RNA was fairly uniformly distributed throughout the gel.

**Analysis of classes of mRNA containing poly A**

In the presence of cycloheximide, second-stage uncoating of vaccinia virus is prevented and mRNA is transcribed from the parental DNA in the cores by the particle-associated RNA polymerase. RNA was extracted from cells pulse labelled 3 h after infection in the
Fig. 2. Analysis of RNA containing poly A synthesized in vaccinia-infected cells in the presence of cycloheximide. Cells were pulse labelled with 50 µCi [3H]-uridine for 20 min at 3 h after infection. (a) RNA was analysed on 3.0 % polyacrylamide gels and run for 4 h at 5 mA per gel. The positions of the [3H]-labelled RK 13 rRNA markers are shown. mRNA species are labelled with their approximate mol. wt. × 10⁶ (see Table 1). (b) RNA was analysed by sucrose velocity sedimentation. The positions of the internal Escherichia coli rRNA markers are indicated.

presence of cycloheximide and the mRNA species containing poly A (‘class 1 early’ mRNA) were examined by electrophoresis in a 3 % polyacrylamide gel (Fig. 2a). They were also analysed by sucrose gradient sedimentation (Fig. 2b). The ranges of sizes obtained by the two techniques were comparable but the resolution on polyacrylamide was far superior. Six discrete peaks were resolved by gel electrophoresis and a number of reproducible shoulders were also observed. The highest mol. wt. peak was at 1.6 × 10⁶ although some material migrated more slowly. It was possible, therefore, that species of even higher mol. wt. were present in small quantities. The mol. wt. of the species were estimated from the positions of the internal rRNA markers and are the means from three independent analyses.

In order to obtain specifically the ‘class 2 early’ mRNA species, infected cells were inhibited with cytosine arabinoside. From the results in Fig. 1 it appeared that by 3 h after infection of inhibited cells the mRNA transcribed was characteristically ‘late’. This implied that second stage uncoating of the virus had occurred by 3 h. If cells are inhibited with cyto-
Fig. 3. Analysis of [\(^{14}\)C]-uridine labelled RNA synthesized in the presence of cycloheximide (●—●) and [\(^{3}\)H]-uridine labelled RNA synthesized in the presence of cytosine arabinoside (■—■). Cells were pulsed with 12·5 µCi \([^{14}\text{C}]\)- or 100 µCi \([^{3}\text{H}]\)-uridine. Extraction and analysis on 3 % polyacrylamide gels was as described in Fig. 2(a).

Fig. 4. Analysis of RNA containing poly A synthesized in vaccinia-infected cells at 6 h after infection. Extraction and analysis on 3 % polyacrylamide gels was as described in Fig. 2(a).

sine arabinoside then 'late' events are not expressed and by 3 h it would be expected that the 'class 1 early' transcription would be terminated by second stage uncoating. Transcription of RNA at this time would therefore be of predominantly the 'class 2 early' type. Cells were therefore pulse labelled at 3 h in the presence of cytosine arabinoside and the synthesized mRNA species containing poly A were extracted and analysed in a 3 % polyacrylamide gel. The profile obtained with the 'class 2 early' poly A-containing mRNA was almost identical to that obtained with the 'class 1 early' material shown in Fig. 2(a).

A comparison was made of the classes of 'early' mRNA using two distinguishable radioactive isotopes. 'Class 1 early' species were labelled with \([^{14}\text{C}]\)-uridine and 'class 2 early' with \([^{3}\text{H}]\)-uridine. These mRNA species containing poly A were then run together in a 3 % polyacrylamide gel. The results in Fig. 3 demonstrate a remarkable similarity in
Table 1. Hybridization of labelled RNA with denatured DNA immobilized on cellulose nitrate filters

<table>
<thead>
<tr>
<th>Class of poly A mRNA to be hybridized</th>
<th>Input (ct/min)</th>
<th>DNA type immobilized on filter</th>
<th>ct/min hybridized</th>
<th>% mRNA hybridized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1 early</td>
<td>5000</td>
<td>Vaccinia</td>
<td>228</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RK</td>
<td>32</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calf thymus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Class 2 early</td>
<td>5800</td>
<td>Vaccinia</td>
<td>296</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RK</td>
<td>98</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calf thymus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>2</td>
<td>0.03</td>
</tr>
<tr>
<td>Late</td>
<td>9000</td>
<td>Vaccinia</td>
<td>525</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RK</td>
<td>35</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calf thymus</td>
<td>2</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>5</td>
<td>0.06</td>
</tr>
</tbody>
</table>

both the sizes and quantities of the RNA species in the two classes of ‘early’ mRNA. Small differences were occasionally seen in double-label runs (Fig. 3 in the region of fraction 33 to 35) but none was observed consistently and these may have been due to errors in counting.

A sample of mRNA containing poly A made ‘late’ in the infection (6 h) was also analysed on a 3 % polyacrylamide gel. No resolution of individual species was obtained (Fig. 4), implying a very heterogeneous population of RNA species. The gel profile confirmed that the ‘late’ species containing poly A were larger than the ‘early’ species, the peak indicating a mol. wt. of 1.2 to 1.4 x 10^6.

Molecular hybridization

To obtain direct evidence that the mRNA species containing poly A were coded for by the virus genome, the ability of the labelled RNA to hybridize to denatured DNA immobilized on cellulose nitrate filters was tested. DNA samples from vaccinia virus, RK 13 cells and calf thymus were used and the blank values were obtained using filters with no bound DNA. The results are shown in Table 1. They indicate that in all three cases the majority of the RNA produced during the times of the pulses had sequences characteristic of the vaccinia genome. There was a minority of sequences present (6 to 25 %) which bound to the host DNA and these may have represented low levels of host mRNA which were synthesized and transported to the cytoplasm during the pulses.

DISCUSSION

The size ranges of mRNA species containing poly A synthesized at various stages of the infection of rabbit kidney cells with vaccinia virus have been examined by polyacrylamide gel electrophoresis. The RNA synthesized 1.5 h after infection was heterogeneous in size and the profile on a 2.4 % polyacrylamide gel showed a peak at a mol. wt. of approx. 0.5 x 10^6. RNA synthesized at 3 h and later was considerably larger with a peak in the region 1.2 to 1.4 x 10^6. Several other groups of workers who have analysed the sizes of poxvirus mRNA species by sucrose velocity sedimentation (Salzman et al. 1964; Oda & Joklik, 1967) have observed a similar increase in size of the mRNA species synthesized at ‘late’ times, although in these cases species containing poly A were not selected specifically.

The results obtained by Kates & McAuslan (1967) suggested that there were differences
between the 'class 1 early' and 'class 2 early' mRNA species synthesizes in vaccinia-infected cells. As this would provide an interesting transcriptional control mechanism, we investigated this possibility using 3 % polyacrylamide gels to obtain increased resolution of the messengers containing poly A. The results were striking in that the species transcribed from the cores before second stage uncoating (class 1) were indistinguishable by electrophoresis from those synthesized after second stage uncoating (class 2). In double-label experiments occasional differences were seen (Fig. 3) but these were not reproducible. Essential controls were included in these experiments to check that no degradation of the RNA species occurred during their preparation.

As the mechanism of binding of mRNA species containing poly A to cellulose nitrate filters is somewhat obscure, we confirmed that we were looking at a species containing poly A by analysing the 'class 1 early' mRNA which specifically bound to oligo-dT cellulose (Searle) (Aviv & Leder, 1972). The profiles obtained with mRNA which bound to oligo-dT cellulose were identical to those obtained with mRNA which bound to filters, thus confirming the specificity of the cellulose nitrate for species containing poly A sequences.

Approximate mol. wt. of the 'early' mRNA species containing poly A were calculated from the positions of the internal rRNA markers and these are shown in Table 2. If it is assumed that each RNA species is a monocistronic message then the 'early' mRNA would code for polypeptides in the mol. wt. range 28000 to 210000. In a recent study of the virus-specified polypeptides synthesized in vaccinia-infected cells, the largest polypeptide detected at 'early' times had a mol. wt. of 125000 (Esteban & Metz, 1973). This raises the possibility that the polypeptide products (210000 and 160000 respectively) of the RNA species of mol. wt. $1.6 \times 10^6$ and $1.2 \times 10^6$ may be cleaved rapidly to lower mol. wt. material, a process known to occur in vaccinia-infected cells (Pennington, 1973). Alternatively, the RNA may be processed before translation, either losing a non-functional portion of the molecule or undergoing cleavage to two or more functional messenger species.

It is possible that the peaks we have observed by polyacrylamide electrophoresis in 3 % gels were each composed of a cluster of mRNA species of similar mol. wt. Approximate calculations based on the assumption that the mol. wt. of vaccinia DNA is $160 \times 10^6$ (Sarov & Becker, 1967) have shown that the proportion of the genome required to code for

### Table 2. Early mRNA species in vaccinia-infected cells

<table>
<thead>
<tr>
<th>Early mRNA species (approximate mol. wt. $\times 10^{-6}$)*</th>
<th>Coding potential (mol. wt. polypeptide $\times 10^{-2}$)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1.6</td>
<td>210</td>
</tr>
<tr>
<td>B 1.2</td>
<td>160</td>
</tr>
<tr>
<td>C 0.91‡</td>
<td>120</td>
</tr>
<tr>
<td>C 0.79</td>
<td>105</td>
</tr>
<tr>
<td>D 0.59‡</td>
<td>78</td>
</tr>
<tr>
<td>D 0.51</td>
<td>68</td>
</tr>
<tr>
<td>D 0.35‡</td>
<td>47</td>
</tr>
<tr>
<td>D 0.32</td>
<td>43</td>
</tr>
<tr>
<td>E 0.21</td>
<td>28</td>
</tr>
</tbody>
</table>

* Calculation of mol. wt. is based on the mobility of the species relative to the internal rRNA markers.
† The mol. wt. of a triplet codon is assumed to be 900 and that of a typical amino acid 120. Coding potential = [(mol. wt. RNA species)/900] x 120.
‡ These RNA species appeared as unresolved shoulders on the gel profiles.
either the 'class I early' or 'class 2 early' mRNA species containing poly A observed is about 9%. Kates & Beeson (1970) estimated from in vitro studies that 'class I early' mRNA is transcribed from approx. 14% of the virus genome. As these authors examined the total mRNA population and did not attempt to select the poly A messengers, it is likely that we are approaching the level of resolution at which individual mRNA species containing poly A may be resolved.

Very poor resolution of the 'late' mRNA species was obtained by polyacrylamide electrophoresis. The peak on the gel was of mol. wt. 1.2 to 1.4 x 10^6 and material larger than the mol. wt. 1.6 x 10^6 component was resolved at 'early' times. The failure to resolve species at 'late' times was probably due to the large number of species transcribed at this time.

Our hybridization results confirmed that the majority of the RNA species examined were virus-specified, but there was a small proportion of sequences present in the mRNA population which were host-specified. The most likely explanation for this would seem to be that a small amount of host mRNA was synthesized in the nucleus during the pulses and escaped to the cytoplasm before the end of the labelling period.

The demonstration that a particular mRNA species is transcribed at a particular time does not necessarily mean that it is also translated. We therefore hope to extend this work to examine the species of mRNA associated with the polysomes in infected cells to obtain information on translational controls.

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REFERENCES


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