Inhibition of Protein Synthesis by Vaccinia Virus. II. Studies on the Role of Virus-induced RNA Synthesis

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SUMMARY

Cytoplasmic RNA synthesis can be detected in vaccinia virus-infected HeLa cells in the presence of 2 μg/ml but not 20 μg/ml of actinomycin D. When RNA synthesis is observed protein synthesis is inhibited in infected, treated cells. We had previously noted that such a correlation may also be observed in infected, cycloheximide-treated cells. If actinomycin D (20 μg/ml) is added to these cells at various times after infection and treatment, the inhibition of protein synthesis seen upon removal of cycloheximide does not continue beyond the point to which it had developed before the actinomycin D was added. These results indicate that the inhibition of protein synthesis can be correlated with the amount of cytoplasmic RNA synthesized in infected cells and that this RNA synthesis and the subsequent inhibition of protein synthesis can be prevented by sufficiently high concentrations of actinomycin D. The cytoplasmic RNA which is synthesized does not appear to consist of double-stranded RNA nor of extensive self complementary regions. The cytoplasmic RNA synthesized in infected, cycloheximide treated cells appears to consist of early virus mRNA which can function as mRNA in vitro in a cell-free system derived from normal cells. An examination of the phosphorylation of ribosomal proteins shows six additional phosphoproteins in infected cells, two of which may be observed in infected cycloheximide-treated cells, suggesting that phosphorylation of ribosomal proteins cannot be directly correlated with the inhibition of overall protein synthesis seen in infected cycloheximide-treated cells.

INTRODUCTION

Host protein synthesis is sharply inhibited by vaccinia virus infection (Kit & Dubbs, 1962; Shatkin, 1963; Holowczak & Joklik, 1967; Salzman & Sebring, 1967). Two hypotheses have been advanced to explain this inhibition. The first suggests that the inhibition of protein synthesis in vaccinia-infected HeLa cells was the result of a component of the virion (Moss, 1968). Infection of cells (25, 50 or 100 p.f.u./cell) in the presence of actinomycin D (5 μg/ml) resulted in multiplicity dependent inhibitions of protein synthesis. Furthermore, cells infected with 25 p.f.u./cell in the presence of both actinomycin D (5 μg/ml) and cycloheximide (300 μg/ml) failed to resume protein synthesis upon removal of the drug. This, with the observation that u.v.-inactivated virus also inhibited protein synthesis, led to the conclusion that a virion component was responsible for the inhibition (Moss, 1968). The

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second hypothesis states that the inhibition of protein synthesis can be correlated with virus-induced RNA synthesis (Bablanian, 1975; Bablanian et al. 1978). It was found that while vaccinia-infected (300 particles/cell), cycloheximide-treated (300 μg/ml), L and HeLa cells failed to resume protein synthesis upon removal of the drug, similarly infected LLC-MK2 cells did resume protein synthesis upon cycloheximide reversal. We have prepared cell-free amino acid incorporating systems from such infected cells in which the inhibition of protein synthesis observed in vivo may be reproduced in vitro (Schrom & Bablanian, 1979).

It was also determined that the onset and extent of inhibition of protein synthesis in vaccinia-infected cells correlates well with the rate of cytoplasmic virus RNA synthesis in infected cells. HeLa and L cells show an increasing rate of cytoplasmic RNA synthesis while in LLC-MK2 cells the rate of RNA synthesis is lower and does not increase with time. The host dependent difference emphasizes the importance of the cell in a virus–cell interaction (Bablanian, 1975; Bablanian et al. 1978).

The use of inhibitors of RNA synthesis has led to conflicting conclusions on the requirement for RNA synthesis in the establishment of inhibition of protein synthesis. Moss (1968) found that 5 μg/ml of actinomycin D could not prevent the inhibition of protein synthesis. Previous work in our laboratory showed that 20 μg/ml of actinomycin D could prevent the inhibition of protein synthesis (Bablanian et al. 1978). We did, however, in agreement with the observations of Moss (1968), find that lower concentrations of actinomycin D (1 μg/ml) allowed the development of inhibition of protein synthesis. Previous work has demonstrated that, in the presence of 1 μg/ml of actinomycin D, early virus RNA synthesis occurs in infected L cells (Metz & Esteban, 1972). Furthermore, Rosemond-Hornbeak & Moss (1975) demonstrated that a small poly(A)-rich RNA is made and thus may contribute to the virus-induced inhibition of host polypeptide synthesis. In order to clarify this situation we have examined RNA synthesis in vaccinia-infected cells, treated with various concentrations of actinomycin D. Our results suggest that higher concentrations of actinomycin D may be necessary to penetrate the virus cores in order to effectively inhibit DNA-dependent RNA synthesis. Our previous work (Bablanian, 1975; Bablanian et al. 1978) has indicated that, in cells infected with vaccinia virus in the presence of cycloheximide, the rate of cytoplasmic RNA synthesis correlates well with the inhibition of protein synthesis. We have further examined this phenomenon and will present evidence to show that the inhibition is also related to the amount of RNA synthesized.

It is not known what kind of cytoplasmic RNA is responsible for the inhibition nor how it acts. There is ample evidence that dsRNA can inhibit protein synthesis (Hunt & Ehrenfeld, 1971; Robertson & Mathews, 1973; Hunter et al. 1975; Lebleu et al. 1976; Roberts et al. 1976). It is also known that such a molecule is synthesized in vaccinia-infected cells (Colby & Duesberg, 1969; Duesberg & Colby, 1969; Colby et al. 1971). However, it is not known whether the synthesis of such a molecule can be correlated with the inhibition of protein synthesis. Evidence will be presented in this communication which indicates that dsRNA is not synthesized in substantial amounts under conditions where inhibition of protein synthesis may be observed. The inhibition of protein synthesis by dsRNA has been shown to be mediated by phosphorylation of initiation factor IF-3 (eIF-2) (Kaempfer & Kaufman, 1973). We have examined the phosphorylation of ribosomal proteins in vaccinia-infected cyclo-heximide-treated cells and will present evidence which suggests that such a process is not involved in the inhibition of overall protein synthesis.

**METHODS**

*Virus and cell cultures.* The growth of vaccinia virus (strain WR) and the maintenance of HeLa and L-929 monolayers was as described previously (Bablanian et al. 1978).

*Measurement of protein synthesis* in vivo. Protein synthesis in infected cells was measured
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as described previously (Bablanian et al. 1978). Protein determinations are carried out according to Bramhall et al. (1969) and hot trichloroacetic acid (TCA)-insoluble incorporation is measured according to Mans & Novelli (1961).

Measurement of RNA synthesis in vivo. The rate of RNA synthesis was determined by the method of Becker & Joklik (1964), a procedure which assumes that most of the RNA labelled in the cytoplasm of infected cells during a 10 min pulse with 3H-uridine is from the virus.

Polyacrylamide gel electrophoresis (PAGE). 35S-methionine-labelled proteins were analysed by PAGE in a vertical gel slab apparatus. Resolving gels 150 mm long and 1.5 mm thick and containing gradients of acrylamide monomer of 5 to 15% were cast using the buffer system of Laemmli (1970). Electrophoresis was at 120 V until the tracking dye reached the bottom of the gel. The gel was washed in 7% acetic acid for several hours and dried under vacuum and autoradiographed (Fairbanks et al. 1965) using DuPont Cronex 2 X-ray film.

Measurements of double-stranded RNA in infected cells. Cells were infected or mock-infected in the presence of 20 μCi/ml of 3H-uridine. At 3.5 h p.i. cytoplasmic RNA was extracted by a modification of the method of Oda & Joklik (1967). Cells were washed with cold RSB (10 mM-tris-HCl, pH 7.6; 10 mM-KCl; 1.5 mM-MgCl2), scraped into 2 ml of RSB and disrupted by 20 strokes of a Dounce homogenizer. The homogenates were centrifuged at 2000 rev/min for 10 min and 10% SDS was added to the supernatant to 0.5%. After 3 to 5 min at 37 °C, sodium acetate (pH 5.0) was added to 0.1 M. Sodium perchlorate and ethylene diamine tetra-acetic acid (EDTA) were added to 1 M and 1.5 mM respectively. An equal vol. of chloroform:isoamyl alcohol (24:1) was added and, after mixing, the samples were centrifuged to separate the phases. The aqueous layer was re-extracted one to five times until the interface between the phases remained clear. The RNA was then precipitated at −20 °C overnight by addition of 2 vol. of ethanol. The RNA was collected by centrifugation and dissolved in 200 μl of TNE buffer (10 mM-tris-HCl pH 7.6, 0.1 M NaCl, 1 mM-EDTA). Portions (10 μl) of this RNA were precipitated with 1 ml of 75% TCA after addition of 100 μg carrier yeast RNA. For ribonuclease treatment 100 μl samples were diluted to 0.5 ml in TNE and 20 μg/ml of bovine pancreatic ribonuclease (Worthington) dissolved in 4× SSC (150 mM-NaCl, 15 mM-sodium citrate, 1 mM-EDTA–SSC) was added. After 30 min at 37 °C, 100 μg of carrier RNA and 2 ml of 75% TCA was added. The precipitates were collected on Whatman GF/C filters after 30 min at 4 °C, dried and counted. Self annealing was performed by bringing the samples to the concentration of 4× SSC and incubating at 65 °C for 18 h. A sample was removed to measure total acid-precipitable radioactivity and the balance was treated with ribonuclease as described. The counts were corrected to a constant vol. of 100 μl.

Examination of phosphorylation of ribosomal proteins in infected cells. L-929 cells were infected (300 particles/cell) or mock-infected in the presence or absence of 300 μg/ml of cycloheximide. The infection was carried out in reinforced Eagles' medium (Bablanian et al. 1965) from which phosphate had been omitted and in the presence of 2% dialysed foetal bovine serum. The cells were labelled from the start of the infection with 50 μCi/ml of 32PO4 (carrier-free, The Radiochemical Centre, Amersham, Bucks, U.K.). At 3.5 h p.i. the cells were washed with cold 30 mM-tris-HCl, pH 7.4; 140 mM-NaCl and then with hypotonic medium (10 mM-tris-HCl, pH 7.4, 10 mM-KCl, 1.5 mM-magnesium acetate, 7 mM-2-mercaptoethanol). The cells were drained nearly dry and scraped into what remained of the buffer. After 5 min the cells were homogenized by 20 strokes of a glass Dounce homogenizer. The homogenate was brought to 30 mM-tris-HCl (pH 7.4), 90 mM-KCl, 3.5 mM-magnesium acetate and 7 mM-2-mercaptoethanol and centrifuged at 10000 g for 10 min. Half of the resulting supernatant was made 0.5 M-KCl by dropwise addition of 4 M-KCl. Both the KCl washed and unwashed preparations were centrifuged for 60 min at 50000 rev/min in the
Beckman type 65 rotor. The resulting pellets were subjected to PAGE and autoradiography as described above.

Preparation of cell-free amino acid-incorporating system. Cell-free amino acid-incorporating systems were prepared from L cells grown in suspension. The cells were washed three times with cold tris-saline (30 mM-tris-HCl, pH 7.4; 140 mM-NaCl) and suspended in 1.5 packed cell vol. of hypotonic medium (10 mM-tris-HCl, pH 7.4; 10 mM-KCl; 1.5 mM-magnesium acetate; 7 mM-2-mercaptoethanol). After 5 min the cells were homogenized by 20 strokes of a glass Dounce homogenizer. The homogenate was brought to 30 mM-tris-HCl (pH 7.4); 90 mM-KCl; 3.5 mM-magnesium acetate and 7 mM-2-mercaptoethanol and centrifuged at 10,000 g for 10 min.

The supernatants were pre-incubated at 37 °C for 40 min after addition of adenosine triphosphate (ATP; 1 mM), guanosine triphosphate (GTP; 0.1 mM), cytidine triphosphate (CTP; 0.6 mM), creatine phosphate (10 mM), creatine kinase (0.16 mg/ml) and 40 mM each of all of the amino acids (all obtained from Calbiochem, La Jolla, Calif., U.S.A.). The extract was chilled and desalted by passage through Sephadex G25 columns prior to storage and assay. Sephadex columns were prepared by equilibration of the gel in 30 mM-tris-HCl (pH 7.4); 100 mM-KCl; 5 mM-magnesium acetate; 7 mM-2-mercaptoethanol and 5% (v/v) glycerine. The extract (5 μl) was passed through the column and the fractions containing the greatest amount of protein (monitored visually by turbidity), corresponding to the void vol., were pooled and stored at −70 °C in 100 μl samples.

Assay of cell-free protein synthesis directed by RNA extracted from infected cells. The RNA extracted from infected cells as described above was added at 3 μg per assay to cell-free protein synthesizing systems prepared as described above. The reaction mixture contained, in a total vol. of 50 μl, tris-HCl (pH 7.4); 35 mM; 2-mercaptoethanol; 9 mM; ATP; 1 mM; GTP; 0.1 mM; CTP; 0.6 mM; creatine phosphate; 10 mM; creatine kinase; 0.16 mg/ml; 35S-methionine; 100 μCi/ml; 40 μM each of the other 19 amino acids; 10 μl of the appropriate S10; 50 mM-KCl and 3.5 mM-magnesium acetate. For endogenous mRNA in non-pre-incubated extracts KCl was usually 110 mM and magnesium acetate 4.5 mM, unless otherwise indicated. L cell mRNA and globin mRNA were translated at 50 mM-KCl and 3.5 mM-magnesium acetate. The reaction was carried out at 30 °C for 120 min. Ten μl samples were removed and placed on filter paper discs (Whatman no. 42, 2.54 cm) which had previously been spotted with 1% (w/v) casamino acids. When all of the samples had been collected the papers were dried and hot TCA-insoluble radioactivity was measured as described above. The remaining portion of the reaction mixture was mixed with an equal vol. of electrophoresis sample buffer for polyacrylamide gel electrophoresis and autoradiography.

RESULTS

RNA synthesis in vaccinia-infected cells in the presence of actinomycin D

Previous studies using actinomycin D to inhibit vaccinia virus RNA synthesis (Moss, 1968; Bablanian, 1975; Rosemond-Hornbeak & Moss, 1975; Bablanian et al. 1978) have left unresolved the question of whether RNA synthesis is required for establishment of inhibition of protein synthesis in vaccinia-infected cells. In order to clarify this question HeLa cells were infected with 900 particles per cell in the presence of 2 or 20 μg/ml of actinomycin D. At various times after infection cytoplasmic RNA synthesis and protein synthesis were measured. Fig. 1 shows that RNA synthesis as measured by uridine incorporation, can be detected in the presence of 2 μg/ml but not 20 μg/ml actinomycin D. When protein synthesis is examined 3.5 h p.i. (Fig. 2) it can be seen that 2 μg/ml actinomycin D is not sufficient to prevent the virus-induced inhibition of protein synthesis. However, when
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Fig. 1. Cytoplasmic RNA synthesis in vaccinia virus-infected cells in the presence of different concentrations of actinomycin D. HeLa cells were infected with 900 particles/cell of vaccinia virus in the presence of 20 μg/ml or 2 μg/ml actinomycin D or in the presence of 300 μg/ml cycloheximide. At the indicated times p.i. the cells were pulse-labelled with 3H-uridine and cytoplasmic RNA synthesis was measured as described in the Methods. The background incorporation in treated uninfected cells has been subtracted. •—• Cells infected in the presence of 20 μg/ml actinomycin D; ▲—▲, cells infected in the presence of 2 μg/ml actinomycin D; ■—■; cells infected in the presence of cycloheximide.

cells are infected in the presence of 20 μg/ml actinomycin D the inhibition of protein synthesis is largely prevented. From this we conclude that when sufficient concentrations of actinomycin D are present, the synthesis of virus-induced RNA is prevented and protein synthesis is not inhibited.

It should be noted that, in agreement with the results of others (Rosemond-Hornbeak & Moss, 1975), we have noted incorporation of 3H-adenosine even in the presence of 20 μg/ml of actinomycin D which is consistent with the observation that polyadenylation of vaccinia mRNA is not inhibited by actinomycin D (Kates & Beeson, 1970).

Relationship between amount of cytoplasmic RNA synthesized in infected cells and inhibition of protein synthesis

Our previous results (Bablanian et al. 1978) have shown that when HeLa cells are infected with vaccinia virus in the presence of cycloheximide the rate of RNA synthesis increases with time and this increase in the rate is related to the inhibition of protein synthesis. It is possible that this inhibition is related to the amount of RNA present or to an effect which develops with time due to the presence of a specific class of early RNA. In order to distinguish these possibilities HeLa cells were infected in the presence of cycloheximide and,
Fig. 2. Effect of actinomycin D and cycloheximide on virus and host polypeptide synthesis in vaccinia virus-infected HeLa cells. Cells were infected with 900 particles/cell or infected-treated in the presence of actinomycin D (ACD) or cycloheximide. At 3 h p.i. the cells were washed free of cycloheximide and all cultures were labelled for 30 min with $^{35}$S-methionine. PAGE and autoradiography were performed as described in the Methods. 1, Virus + 20 µg/ml ACD; 2, 20 µg/ml ACD; 3, virus + 2 µg/ml ACD; 4, 2 µg/ml ACD; 5, virus + 300 µg/ml cycloheximide; 6, 300 µg/ml cycloheximide; 7, virus; 8, cells.
Fig. 3. Relationship between amount of cytoplasmic RNA synthesized in infected cells and inhibition of protein synthesis. HeLa cells were infected (900 particles/cell) in the presence of 300 μg/ml cycloheximide. Replicate cultures were treated with 20 μg/ml actinomycin D at 60, 120 or 180 min p.i. At the indicated times p.i., the cells were washed free of cycloheximide, labelled with 35S-methionine for 30 min and sp. act. was determined as described in the Methods. Results are expressed as percentage of uninfected control. ●—●, No actinomycin D; actinomycin D added at: ■—■, 60 min; ▲—▲, 120 min; ▼—▼, 180 min.

at various times p.i., 20 μg/ml actinomycin D was added to stop further RNA synthesis. The cycloheximide was removed from treated cultures and protein synthesis was measured as described in the Methods.

Fig. 3 shows that when RNA synthesis is interrupted by the addition of actinomycin D the inhibition of protein synthesis does not progress beyond the point to which it had been reduced before the addition of the drug. This suggests that the inhibition of protein synthesis is related to the amount of RNA synthesized and not to a catalytic effect produced by an early RNA species. What remains unclear is what RNA is responsible for this inhibition.

Relationship of dsRNA to inhibition of protein synthesis in infected cells

The one RNA species which has been shown to be an inhibitor of protein synthesis is double-stranded RNA (Hunt & Ehrenfeld, 1971; Robertson & Mathews, 1973; Hunter et al. 1975; Lebleu et al. 1976; Roberts et al. 1976). This molecule has also been shown to be synthesized in vaccinia-infected cells (Colby & Duesberg, 1969; Duesberg & Colby, 1969; Colby et al. 1971), however, it has not been shown that the synthesis of dsRNA may be specifically correlated with the inhibition of protein synthesis in infected cells. L-929 cells were infected or mock-infected in the presence or absence of cycloheximide and in the presence of 3H-uridine. At 3.5 h p.i. total cytoplasmic RNA was extracted and the total and ribonuclease-resistant radioactivity was measured. It has previously been shown (Kates & McAuslan, 1967) that early virus RNA synthesis proceeds at an accelerated rate in the presence of cycloheximide. Table 1 shows that while we also observe a large increase in the amount of acid-precipitable radioactivity found in infected treated cells, the percentage of this which is ribonuclease resistant does not increase in infected treated cells. In the absence of cycloheximide, infected cells show an increase in the percentage of nuclease-resistant material. The infected cycloheximide-treated cells show an increase in the absolute amount of ribonuclease-resistant RNA. Although it is possible that this does represent the synthesis of small amounts of dsRNA, and despite the fact that nuclease resistance is not an absolute criterion for double-strandedness, we suggest that the failure of this material
Table 1. **Cytoplasmic RNA* resistant to ribonuclease (RNase) from vaccinia-infected L-929 cells**

<table>
<thead>
<tr>
<th>RNA Cells</th>
<th>Cycloheximide</th>
<th>Total $^3$H-uridine (ct/min)</th>
<th>RNase-resistant % resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>-</td>
<td>15710</td>
<td>387</td>
</tr>
<tr>
<td>Infected</td>
<td>+</td>
<td>99350</td>
<td>773</td>
</tr>
<tr>
<td>Uninfected</td>
<td>-</td>
<td>37160</td>
<td>239</td>
</tr>
<tr>
<td>Uninfected</td>
<td>+</td>
<td>34030</td>
<td>257</td>
</tr>
</tbody>
</table>

* Cells were infected or mock-infected in the presence or absence of 300 $\mu$g/ml cycloheximide and in the presence of 20 $\mu$Ci/ml $^3$H-uridine. After 3.5 h total cytoplasmic RNA was extracted and treated with ribonuclease as described in the Methods.

Table 2. **Self annealing* of cytoplasmic RNA from vaccinia-infected L-929 cells**

<table>
<thead>
<tr>
<th>RNA Cells</th>
<th>Cycloheximide</th>
<th>Total $^3$H-uridine (ct/min)</th>
<th>RNase-resistant % resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>-</td>
<td>15205</td>
<td>295</td>
</tr>
<tr>
<td>Infected</td>
<td>+</td>
<td>98032</td>
<td>1392</td>
</tr>
<tr>
<td>Uninfected</td>
<td>-</td>
<td>37084</td>
<td>472</td>
</tr>
<tr>
<td>Uninfected</td>
<td>+</td>
<td>32581</td>
<td>456</td>
</tr>
</tbody>
</table>

* Total cytoplasmic RNA, prepared as described in Table 1, was self-annealed in 4 x SSC for 18 h at 65 °C before RNase treatment.

It is possible that there may exist in infected cells complementary RNA sequences which have not formed double-stranded molecules. In order to exclude this possibility the extracted RNA was self annealed for 18 h at 65 °C in 4 x SSC and again subjected to ribonuclease digestion. Again the only sample which shows an increased nuclease resistance is the infected untreated cells (Table 2). This is in agreement with the observations of Boone & Moss (1978) that early vaccinia RNA does not contain extensive self complementary sequences. These results suggest that the extensive inhibition of protein synthesis we observe under these conditions cannot be correlated with the synthesis of large amounts of double-stranded RNA. However, in view of the approximate doubling of the absolute amount of nuclease-resistant RNA in the infected-treated cells, the involvement of dsRNA cannot be definitely excluded.

It is possible that small amounts of dsRNA functioning in a catalytic fashion (Hunter et al. 1975) might act to inhibit protein synthesis. One situation in which this has been shown to occur is through the phosphorylation of IF-3 (eIF-2) (Kaempfer & Kaufman, 1973). It is known that dsRNA stimulates phosphorylation of ribosomal proteins in interferon-treated cells (Roberts et al. 1976) and also that ribosomal proteins are phosphorylated in vaccinia-infected cells (Kaerlein & Horak, 1976). It was therefore of interest to examine the phosphorylation of ribosome-associated proteins in infected treated cells.

**Role of protein phosphorylation in the inhibition of protein synthesis**

L-929 cells were infected or mock-infected in the presence or absence of cycloheximide and in the presence of 50 $\mu$Ci/ml $^{32}$P-H$_3$PO$_4$. At 3.5 h p.i. crude unwashed ribosomes and KCl washed ribosomes were prepared as described in the Methods and analysed by PAGE.
Fig. 4. Phosphorylation of ribosomal proteins in vaccinia-infected cells. L-929 cells were infected or mock-infected (300 particles/cell) in the presence or absence of 300 μg/ml cycloheximide and in the presence of 50 μCi/ml ³²PO₄ in phosphate-free REM. At 3.5 h p.i. unwashed and KCl washed ribosomes were prepared as described in the Methods and subjected to PAGE and autoradiography. 1, unwashed infected ribosomes; 2, unwashed infected-cycloheximide treated ribosomes; 3, unwashed uninfected ribosomes; 4, unwashed uninfected-cycloheximide treated ribosomes; 5, KCl washed infected ribosomes; 6, KCl washed infected-treated ribosomes; 7, KCl washed uninfected ribosomes; 8, KCl washed uninfected-cycloheximide treated ribosomes. The additional phosphorylated bands are indicated at their left and identified by letters. The approx. mol. wt. range was from 45000 to 10000.
Table 3. Addition of total cytoplasmic RNA from vaccinia-infected L-929 cells to cell-free protein-synthesizing systems

<table>
<thead>
<tr>
<th>Cells from which RNA was extracted</th>
<th>^35S-methionine (ct/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>19851</td>
</tr>
<tr>
<td>Infected</td>
<td>14445</td>
</tr>
<tr>
<td>Infected-cycloheximide treated</td>
<td>72454</td>
</tr>
<tr>
<td>Uninfected</td>
<td>22653</td>
</tr>
<tr>
<td>Uninfected-cycloheximide treated</td>
<td>35154</td>
</tr>
</tbody>
</table>

* Pre-incubated cell-free systems were prepared from normal cells as described in the Methods. RNA was added at 3 µg/50 µl assay.

It can be seen (Fig. 4) that there are six phosphorylated proteins associated with the unwashed ribosomes of the infected untreated cells not found in infected cells. Five of these (Bands, A, C, D, E, F) remain after KCl washing. The infected treated cells contain two of these proteins. The three major phosphorylated bands (Bands B, C, E) in the infected untreated cells are absent in the cycloheximide-treated infected cells. Since the infected treated cells manifest a complete inhibition of protein synthesis in the absence of extensive phosphorylation, we suggest that phosphorylation of ribosome-associated proteins is not involved in the inhibition of protein synthesis. It may be that the two phosphoproteins common to the infected and infected treated cells (Bands A, F) are related to the selective inhibition of cellular protein synthesis but it does not appear that the overall inhibition of protein synthesis can be correlated with an increase in protein phosphorylation in infected cycloheximide-treated cells.

**Addition of RNA from infected treated cells to cell-free amino acid-incorporating systems**

In order to determine whether the increase in cytoplasmic RNA represented messenger RNA, the total cytoplasmic RNA extracted from infected, infected treated, uninfected treated and uninfected cells was added to cell-free amino acid-incorporating systems prepared from normal cells. When RNA from infected treated cells is added to pre-incubated cell-free systems it is capable of directing amino acid incorporation *in vitro* (Table 3) even though mRNA has not been specifically extracted. A slight stimulation is observed with RNA from uninfected treated cells. However, when the cytoplasmic RNAs from infected untreated or uninfected untreated cells were added to cell-free systems, no such stimulation of incorporation was observed. When the products of these reactions were analysed by PAGE (Fig. 5) several bands were seen which do not correspond to the endogenous products. We presume, therefore, that these bands represent virus proteins, the translation of which was directed by virus mRNAs which had accumulated in the presence of cycloheximide.

**DISCUSSION**

The results presented in this communication support the idea that RNA synthesis is required for the inhibition of protein synthesis by vaccinia virus. We have confirmed the results of Shatkin (1963) and Moss (1968) that cellular protein synthesis is inhibited in the presence of actinomycin D at concentrations up to 5 µg/ml. However, we have also shown, as noted by Metz & Esteban (1972) and Rosemond-Hornbeak & Moss (1975), that RNA synthesis can be observed in cells infected in the presence of as much as 5 µg/ml actinomycin D. When sufficient concentrations of the drug are added to completely prevent RNA synthesis (Fig. 1), inhibition of protein synthesis is not observed (Fig. 2). While 20 µg/ml actinomycin D may appear excessive, it may be that lower concentrations of the drug fail
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Fig. 5. Protein synthesis in vitro directed by RNA from infected cells. L-929 cells were infected or mock-infected (300 particles/cell) in the presence or absence of 300 μg/ml of cycloheximide. At 3.5 h p.i. total cytoplasmic RNA was extracted and added to pre-incubated cell-free protein-synthesizing systems as described in the Methods. 1, no RNA added; 2, RNA from infected cells; 3, RNA from infected cycloheximide-treated cells; 4, RNA from uninfected cells; 5, RNA from uninfected cycloheximide-treated cells.

to penetrate the virus core structure as suggested by Metz & Esteban (1972). The lower concentrations may be sufficient to interfere with RNA synthesis so that the product is aberrant and non-translatable. This would be consistent with the observations of Rosemond-Hornbeak & Moss (1975) that small aberrant poly(A)-rich RNA molecules are synthesized.
in the presence of 5 μg/ml actinomycin D. This sort of molecule may be responsible for the inhibition of protein synthesis in the presence of actinomycin D. However, inhibition of protein synthesis also occurs in the absence of actinomycin D (i.e. in the presence of cycloheximide) therefore other mechanisms of inhibition should be postulated.

We have previously observed a relationship between RNA synthesized in the presence of cycloheximide and inhibition of protein synthesis upon removal of the drug (Bablanian et al. 1978). In the present study we have further examined this relationship. The first approach we took was to determine whether the inhibition is the result of the catalytic action of some RNA species or of the accumulation of an inhibitor which acts in stoichiometric amounts. The results presented in Fig. 3 suggest that the accumulation of RNA is responsible for the development of inhibition of protein synthesis. This accumulation may be interrupted by addition of 20 μg/ml actinomycin D, and once RNA synthesis is interrupted, the inhibition does not advance beyond the point to which it had developed when the actinomycin D was added.

The only situation in which an RNA molecule has been shown to be an inhibitor of protein synthesis is the case of dsRNA (Hunt & Ehrenfeld, 1971; Robertson & Mathews, 1973; Hunter et al. 1975; Lebleu et al. 1976; Roberts et al. 1976). This inhibition of protein synthesis has been shown to be the result of inactivation of an initiation factor (Kaempfer & Kaufman, 1973) which, in reticulocytes and interferon-treated cells, is mediated by phosphorylation (Lebleu et al. 1976; Roberts et al. 1976; Farrell et al. 1977). Although Kaerlein & Horak (1976) have shown, as we have also observed (Fig. 4), that ribosomal proteins are phosphorylated in vaccinia virus-infected cells, our evidence (Table 1, Table 2, Fig. 4) does not suggest the direct involvement of dsRNA or any phosphorylation of ribosomal proteins in the inhibition of protein synthesis by vaccinia virus in infected-cycloheximide treated cells.

The results in Table 3 suggest that the greatest proportion of the RNA synthesized in infected-cycloheximide treated cells represents early virus messenger RNA and it constitutes a large enough fraction of the total RNA content to direct protein synthesis in vitro without oligo(dT) chromatography. It is possible that the accumulation of early mRNA in vast excess may itself interfere with protein synthesis by some as yet unknown process. We have recently presented evidence (Schrom & Bablanian, 1979) which indicates that the inhibition of protein synthesis takes place at the level of initiation of new polypeptide chains, an observation consistent with those of Person & Beaud (1978) and Ben-Hamida & Beaud (1978) obtained by different methods.

We must therefore postulate that early virus mRNA specifically interferes with initiation in infected-cycloheximide treated cells. What cannot be excluded is the possibility that some aberrant RNA, such as, but not necessarily identical to, the small poly (A)-rich molecules observed by Rosemond-Hornbeak & Moss (1975) may be synthesized. However, with our present data, it remains true that the inhibition of protein synthesis in infected treated cells can best be correlated with the synthesis of early virus mRNA.

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