Poly (A) Polymerase Activity in L Cells Following Encephalomyocarditis Virus Infection

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SUMMARY

Poly (A) polymerase activity has been measured in crude cytoplasmic extracts of mouse L cells infected with encephalomyocarditis (EMC) virus. After infection there is first a decrease in enzyme activity followed by an increase which itself precedes detectable virus RNA and protein synthesis. The activity of the enzyme then declines before the release of mature virions and cell death take place. The early inhibition of poly (A) polymerase activity is correlated with the virus-induced shut-off of cellular protein synthesis but it is not due to inhibition of the synthesis of cellular enzyme and occurs in the absence of virus replication. The poly (A) polymerase is not synthesized after infection and modification of its activity can be reversed late in the virus cycle. These results indicate that host poly (A) polymerase activity can be regulated by the virus and further show that there is a correlation between the modification of poly (A) polymerase activity and the biosynthesis of poly (A).

INTRODUCTION

Following reports of the enzymic polymerization of adenylic acids in cells by Edmonds & Abrams in 1957, poly (A) polymerases were found widely distributed in bacteria (August et al. 1962; Gottesman et al. 1962), yeast (Twu & Bretthauer, 1971; Haff & Keller, 1973), animal cells (Edmonds & Abrams, 1960; Venkataaraman & Mahler, 1963; Klemperer, 1965; Giron & Huppert, 1965) and plant cells (Biswas & Biswas, 1966; Walter & Mans, 1970). More recently, a poly (A) polymerase activity has been found associated with some animal viruses (Moss et al. 1973; Stoltzfus et al. 1974). Although these enzymes have been extensively studied, their biological role is still not clearly understood. They are generally assumed to be involved in the post-transcriptional addition of poly (A) to the eukaryotic heterogeneous nuclear RNA, to messenger RNA, and to some virus RNAs (Darnell et al. 1971; Philipson et al. 1971).

Our own previous extensive studies of cytoplasmic poly (A) polymerase from mouse ascites tumour cells (Giron & Huppert, 1972a, b) and results of others (Müller et al. 1975; Müller et al. 1977) failed to provide direct evidence that the regulation, either of synthesis, or of activity of the poly (A) polymerase, actually controls biosynthesis of poly (A) in these cells. To answer this question, a virus-infected cell system (mouse L cells infected with EMC virus) was chosen because in this system the events involved in virus maturation can be synchronized with the time after infection. Hence, by measuring poly (A) polymerase activity at these times, it should be possible to determine whether any correlation exists.
between enzyme activity and virus maturation. EMC virus multiplies exclusively in the
cytoplasm and the virus RNA acts as messenger for the synthesis of virus proteins. The
presence of poly (A) in EMC virus RNA at the 3'OH end has now been clearly demon-
strated by Burness et al. (1975), Porter et al. (1975) and Giron et al. (1976). Early doubts
about its presence may have been due to difficulties related to its relatively small size
compared to that in other picornaviruses (Miller & Plagemann, 1972; Porter et al. 1974).

In this paper, we provide evidence to show that variations in the cellular poly (A) poly-
merase activity accompany the development of virus infection.

METHODS

Infection of cell cultures. Mouse L cells were grown in monolayers (3 x 10^7 cells per
75 cm² Falcon flask) in Eagle's medium (Eurobio) and 10% tryptose phosphate broth
(Difco). Cells were infected with EMC virus at indicated multiplicities of infection (m.o.i.)
using the strain maintained in this laboratory (Sanders et al. 1958). After adsorption at
37 °C for 45 min, infection was allowed to proceed for the indicated times. Cell death was
estimated by eosin staining of cells.

RNA and protein synthesis. At various times after infection, RNA and protein were
labelled simultaneously by the addition of 10 µCi ³H-uridine (sp. act. 21 Ci/mmol) and
0.25 µCi ¹⁴C-labelled amino acids (sp. act. 45 mCi/mAt C) per ml of medium respectively
for 15 min. The radioactivity incorporated into material insoluble in 5% trichloroacetic
acid was determined in Bray's solution in a liquid scintillation spectrometer. Suitable
corrections for channel spill-over were made using a double label technique. Radioactivity
was expressed as ct/min per µg RNA estimated by measuring absorbance at 260 nm or per
µg protein determined by the Lowry method (1951).

Preparation of cytoplasmic extracts and enzymic assay. L cells grown and infected as
already indicated were collected at 4 °C by scraping off the cell sheet in the presence of a
buffer containing 0.001 M-β-mercaptoethanol, 0.001 M-MgCl₂, 0.01 M-tris-HCl, pH 7.4. For
cell lysis, many different techniques were tried such as Dounce disruption and detergent
treatment. In view of the unsatisfactory results obtained, we finally adopted shaking the
cells with glass beads for 2 min at 2000 rev/min. With this technique no nuclei were disrupted
as verified by light microscopy, and the recovery of enzyme activity was already maximum
after the 2 min period.

The assay mixture for poly (A) polymerase contained (in 0.4 ml): tris, pH 8.4, 50 µmol;
MnCl₂, 0.5 µmol; β-mercaptoethanol, 1 µmol; ¹⁴C-ATP, 0.2 µmol (sp. radioactivity
650 000 ct/min/µmol); poly (A) (as a primer), 150 µg; and the enzyme extract. The assays
were performed usually with two protein concentrations (60 and 120 µg) and for 1 or 2 h
at 37 °C to ensure linearity of the enzymic reaction. The reaction was terminated by the
addition of cold 3.5% perchloric acid (PCA) in the presence of 1 mg carrier serum albumin.
After washing twice with cold 3.5% PCA, the precipitate was dissolved in 0.22 M-NH₄OH,
dried and the radioactivity counted in a Tracerlab flow counter. Specific activity of poly (A)
polymerase is expressed as nmol ¹⁴C-AMP incorporated/mg protein/h. The results are
usually expressed as % of uninfected cell activity.

U-¹⁴C-ATP (> 400 mCi/mmol) was purchased from New England Nuclear Corporation;
5-³H-uridine (21 Ci/mmol) and 2, 8-³H-adenosine (30 to 50 Ci/mmol) were purchased from
C.E.A., France. Poly (A) was purchased from Merck, Sharp and Dohme and cycloheximide
from Upjohn.
Poly (A) polymerase activity in EMC infected L cells

Fig. 1. Poly (A) polymerase activity in L cells after infection by EMC virus. L cells were infected with EMC virus at a m.o.i. of 7 to 10. Samples were taken at the end of the adsorption period (0 h) and at different times p.i. Cytoplasmic extracts were prepared and assayed as described in Methods. In parallel, cells were pulse-labelled with 3H-uridine. O-O, Poly (A) polymerase activity; ●-●, 3H-uridine incorporated; □-□, eosin staining of cells.

Table 1. Investigation of the presence of a dissociable inhibitor of poly (A) polymerase activity during the early stage of infection*

<table>
<thead>
<tr>
<th>Control cell extracts</th>
<th>Infected cell extracts</th>
<th>Control extracts + infected cell extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (µg/0.4 ml)</td>
<td>Enzyme activity (µg)</td>
<td>Enzyme activity (µg)</td>
</tr>
<tr>
<td>40</td>
<td>0.61</td>
<td>32</td>
</tr>
<tr>
<td>80</td>
<td>1.57</td>
<td>64</td>
</tr>
<tr>
<td>130</td>
<td>2.33</td>
<td>72</td>
</tr>
<tr>
<td>160</td>
<td>2.62</td>
<td></td>
</tr>
</tbody>
</table>

* See legend to Fig. 1 for details. The poly (A) polymerase activity refers to nmol 14C-AMP incorporated during 1 h at 37 °C. The theoretical values are calculated by adding the amount of 14C-AMP incorporated by each fraction separately.

RESULTS

Poly (A) polymerase activity during virus infection

In earlier experiments, we have shown that L cell cytoplasm shows a poly (A) polymerase activity which displays essentially the same properties, such as requirement for Mn²⁺, poly (A) as initiator and optimum pH, as we described for Krebs ascites cells (Giron & Huppert, 1972b). Infection of L cells by EMC virus leads to a rapid shut-off of cellular RNA and protein synthesis (Martin et al. 1961; Zimmerman et al. 1963). At an m.o.i. of 7 to 10, the appearance of virus components can be detected in the cytoplasm of the cell 3.5 h p.i. Maximum synthesis of virus components is attained between 5 and 6 h and progeny virions are released as lysis and cell death occur (Fig. 1). Poly (A) synthesizing
Fig. 2. RNA synthesis in cells infected with u.v. inactivated virus. L cells were infected with EMC virus at a high m.o.i. Others were inoculated with u.v. inactivated virus (Philips TUV 15 W germicidal tube 1250 erg/mm²). Uninfected cells were treated identically except for the presence of virus. All of the cultures were incubated afterwards in growth medium supplemented with 5 µg/ml of actinomycin D. Samples were harvested at different times after 15 min pulse-labelling with ³H-uridine. □, Uninfected cells; •----•, cells infected with intact virus; ○----○, cells infected with u.v. inactivated virus.

Fig. 3- Poly (A) polymerase activity in the absence of virus synthesis: early effect of infection. Infection was performed at a high m.o.i. under different conditions: ○----○, with intact virus; •----•, in the presence of cycloheximide (7 µg/ml); ■----■, with u.v. inactivated virus; □----□, with heat-inactivated virus.

activity has been measured in cell extracts at 30 or 60 min intervals throughout the infection cycle (Fig. 1). The activity first decreases, reaching a minimum value at about 2-5 to 3 h p.i. (30 to 50% of the value in uninfected cell extracts). It increases later and attains a maximum near 4 h p.i. (60 to 80% of initial levels). The activity of the enzyme declines before the onset of cell lysis. The maximum enzymic activity precedes the maximum of virus RNA synthesis by about 1.5 h and the final decline occurs before that of virus synthesis. The same pattern of enzyme activity is observed in the presence of 5 µg/ml of actinomycin D (results not shown). Numerous experiments were performed and all gave the same results.

At higher m.o.i. (30 or 100), the quantitative variations of enzyme activity remain identical but the successive steps (early decrease, late rise and final decline, in parallel with shortening of the infection cycle) occur about 1 h earlier than under standard conditions.

Poly (A) polymerase activity and shut-off of cellular protein synthesis

We first determined whether the decrease in enzyme activity p.i. was simply due to the shut-off of poly (A) polymerase synthesis. A control experiment was performed in which protein synthesis in non-infected L cells was reduced to less than 5% of the normal level by adding 7 µg/ml of cycloheximide to the medium. The poly (A) polymerase activity persisted at high levels in these cells and slowly decreased with time; in particular, after 10 h, as much as 50% of the initial activity was found. The extent and the rate of the loss of enzyme activity which follows infection cannot therefore be explained by the cessation of enzyme synthesis.
Poly (A) polymerase in EMC infected L cells

Fig. 4. Poly (A) polymerase activity in the absence of virus synthesis: late stages of infection. See legends of Fig. 2 and 3 for details. Infection was performed: ○—○, with intact virus; ■—■, with u.v. inactivated virus; ●—●, in the presence of cycloheximide.

Fig. 5. Evidence that de novo poly (A) polymerase synthesis does not take place after infection. L cells were grown and infected at an m.o.i. of 7. Cycloheximide (7 μg/ml) was added to growth medium for 1 h at the indicated times. Afterwards the cells were washed and supplemented with fresh medium. ○—○, Untreated infected cells; ■—■, cycloheximide present between 2 and 3 h p.i.; □—□, cycloheximide present between 3 and 4 h p.i.

Investigation on the presence of a dissociable inhibitor of poly (A) polymerase activity

To verify the possibility that a virus-induced inhibitor could be responsible for the decrease of poly (A) polymerase activity after infection, we measured the enzyme activity of extracts from non-infected cells alone, or in the presence of increasing quantities of extracts from infected cells prepared 2.5 h and 3 h p.i. As shown in Table I, an additive effect is obtained with both preparations which seems to indicate the absence of any inhibitory factor of poly (A) polymerase activity in infected cells.

Poly (A) polymerase activity in cells where virus replication is blocked

In order to investigate whether all the successive changes in enzyme activity p.i. require virus replication, we measured the poly (A) polymerase activity in cells where no virus synthesis took place.

Early stage of infection

L cells were infected with u.v. irradiated virus in the presence of actinomycin D (4 μg/ml). Virus inactivation was verified by the loss of 5 log of the infectious titre and by the inhibition of 3H-uridine incorporation into acid-insoluble material (Fig. 2). A decline of poly (A) polymerase activity was observed after infection (Fig. 3). However, the relative amounts of enzyme activity were repeatedly higher than in cells infected with intact virus for reasons which are still not clear. Likewise, when cells infected at high m.o.i. were exposed to cycloheximide from the beginning of virus adsorption, a decrease in enzyme activity took place p.i., which was as great as that found in infected cells not treated with the inhibitor (Fig. 3).
On the contrary, infection by heat-inactivated virus (1 h at 60 °C) did not modify the level of enzyme activity.

Later stages of infection

In cells infected with u.v. inactivated virus as described above, the poly (A) polymerase activity increased after the early inhibition period (Fig. 4). Two main differences appeared as compared to cells infected with infectious virus: (1) the maximum amount of enzyme activity was markedly higher, generally exceeding that measured in non-infected cell extracts; (2) the enzyme activity remained at this upper level throughout the experiment. When protein synthesis was inhibited by cycloheximide, the poly (A) polymerase activity increased also, after the initial decrease, up to the level of non-infected control cells (Fig. 4).

Does the rise in poly (A) polymerase activity represent de novo synthesis of enzyme?

The preceding results do not totally exclude the possibility of a virus-induced poly (A) polymerase. Therefore, we used cycloheximide to suppress protein synthesis temporarily: (1) in the interval preceding the increase of enzyme activity, i.e. between 2 and 3 h p.i. (m.o.i. of 7); (2) during the increase itself, from 3 to 4 h p.i. (Fig. 5). Previous studies had established that the effect of the inhibitor is quantitatively reversible. Essentially the same features were observed in both cases: enzyme activity increased in the same way in treated cells as in cells not treated with cycloheximide. Moreover, a second rise in poly (A) polymerase activity appeared at about 6 h (Fig. 5); the earlier the time of drug addition p.i., the higher the level of this second rise in enzyme activity. In cells in which protein synthesis had been inhibited for just 1 h, a second round of virus RNA and protein synthesis took place as soon as the inhibitor was removed and cell death was regularly delayed by 1 to 2 h.
**Effect of protein synthesis inhibition at different times post-infection**

Since it appeared that no poly (A) polymerase synthesis occurs p.i., we considered the regulation of the host enzyme activity throughout the infection cycle. We therefore inhibited protein synthesis in cells infected at high m.o.i. by introducing cycloheximide from 0 to 3 h p.i. As shown in Fig. 6, an important rise in poly (A) polymerase activity, comparable to the levels found in non-infected cell extracts, took place within 30 min regardless of when the inhibitor was introduced. When the cells were exposed to cycloheximide from 0 h of infection, i.e. not including the virus adsorption period, the rise in enzyme activity began earlier than reported above (Fig. 4). This difference is probably due to the synthesis of virus components already taking place during the time (45 min at 37 °C) of virus adsorption.

**Characterization of poly (A) polymerase activity in infected cells**

We examined some properties of the enzyme activity from non-infected and infected cells 2 or 4·5 h p.i. respectively. They appeared very similar in all three extracts: the enzymic reaction is optimum within a wide range of Mn⁺⁺ concentrations (between 0·25 and 1·5 mM) and for two ranges of pH: 8·2 to 8·5 and > 8·9 respectively; the saturating concentration of ATP is 1·5 mM, that of poly (A) 0·2 mM and the thermosensitivity of the enzyme is also identical in extracts of infected and non-infected cells.

**DISCUSSION**

We have described systematic variations in the activity of the poly (A) polymerase in the cytoplasm of L cells following infection by EMC virus. We have found that these variations are independent of the transcription of cellular mRNA as might be expected for picornavirus-infected cells. Although the cytoplasmic extracts were always prepared by a method which apparently preserves the integrity of cell nuclei, we cannot totally exclude the possibility that the observed variations in the poly (A) polymerase activity are due to the release of a nuclear enzyme (Rose et al. 1976) or of other nuclear factors caused by modifications of the nuclear membrane permeability during infection. The initial decrease in poly (A) polymerase activity occurs in the absence of virus synthesis as was shown by following the enzyme activity in cells either infected by u.v. inactivated virus or exposed to cycloheximide. Furthermore, infection by heat-inactivated virus suggests that the protein moiety of the infecting particles is involved in this early effect p.i.

From these characteristics, together with kinetic behaviour and efficiency, we conclude that the enzyme inactivation resembles the virus-induced shut-off of cellular synthesis (Baltimore & Franklin, 1963; Moss, 1968; Bablanian, 1972). The relationship between the enzyme inactivation and the modifications of cellular metabolism which follow virus infection as well as the detailed mechanism involved therein remain to be elucidated. Inhibition of poly (A) polymerase activity was described as early as 1965 (Ortiz et al.) in bacteria, following bacteriophage infection. In this system the synthesis of a protein inhibitor was demonstrated contrary to our own present observations, but the biological significance of this phenomenon has remained unexplained.

The use of the protein synthesis inhibitor cycloheximide showed that no de novo synthesis of poly (A) polymerase takes place during virus infection. We found that the main characteristics of the enzymic reaction remain unchanged p.i. However, increases in enzyme activity and virus multiplication are related since a second round of EMC virus synthesis is accompanied by another rise in poly (A) polymerase activity.

Two observations provide evidence for the stability of poly (A) polymerase in cells,
whether infected or not: (1) the enzyme activity persists at high levels in non-infected cells in which protein synthesis has been suppressed; (2) the virus-induced modifications can be reversed and enzyme activity may return close to the value found in uninfected cells, as late as 3 h p.i. It has been reported for mouse plasmacytoma cells infected by EMC that the cellular RNA polymerases are quantitatively conserved although their activity gradually decreases (Schwartz et al. 1974). The behaviour of both these cellular DNA-dependent RNA polymerases and poly (A) polymerase after infection then contrasts with the high turn-over rate of the virus induced RNA-dependent RNA polymerase typical of picornavirus infection.

While this work was in progress, Korant (1975) reported variations in the activity of proteolytic enzymes in poliovirus-infected Hela cells: the successive changes found were similar to those we described for the poly (A) polymerase, with the exception that a virus-coded protease appears to be neosynthesized after infection.

While studying the length of poly (A) in EMC virus RNA, in cellular mRNA and in virus mRNA, we found that the shut-off of cellular mRNA synthesis during the early stage of infection is accompanied by a modification of their polyadenylation in that there is a gradual decrease in the mean size of the newly made poly (A) chains (Hanania et al. 1976). The present study correlates this shortening with the decrease in poly (A) polymerase activity.

We showed that poly (A) chains 30 to 50 nucleotides long characterize virus RNA molecules present in the polysomes of the cells at 4-5 h p.i., while oligo (A) tracts of only 20 to 25 nucleotides long are located on the virion RNA molecules (Giron et al. 1976). The time of increase of the host poly (A) polymerase activity agrees well with a higher poly (A) content of the virus mRNA. The variations in the level of enzyme activity might be correlated with the availability of primers for polyadenylation. The hypothesis that the activity of this enzyme is regulated by the amount of non-polyadenylated RNAs was discussed by Brakel & Kates (1974) to account for the vaccinia virus-induced poly (A) polymerase in HeLa cells.

We have suggested (Hanania et al. 1976) that in EMC virus-infected cells the extent of polyadenylation could play some part in the fate of virus RNA molecules and control virus infection. Proteolytic degradation has also been proposed to regulate several virus functions (Korant, 1975). It is interesting to note that both the poly (A) polymerase and the protease activities undergo specific virus-induced alterations after infection.

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REFERENCES


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