Replication of Mengovirus

II. General Properties of the Viral-Induced Ribonucleic Acid Polymerase

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ABSTRACT

PLAGEMANN, PETER G. W. (Western Reserve University, Cleveland, Ohio), AND H. EARLE SWIM. Replication of mengovirus. II. General properties of the viral-induced ribonucleic acid polymerase. *J. Bacteriol.* **91**:2327-2332. 1966—Mengovirus induces the appearance of a ribonucleic acid (RNA) polymerase activity in Novikoff hepatoma cells which is readily distinguished from the deoxyribonucleic acid (DNA)-dependent RNA polymerase since it is not inhibited by actinomycin D or deoxyribonuclease, but is inhibited by ammonium sulfate, and is stable at -17°C . The incorporation of uridine into RNA by infected cells in the presence of actinomycin D does not reflect the viral polymerase activity as measured in cell-free preparations. The viral-induced RNA polymerase is produced in a biphasic fashion. Puromycin inhibits the production of viral polymerase, and in its presence the enzyme appears to be unstable between 4 and 6 hr. Puromycin also prevents the secondary rise in polymerase which begins at the end of replicative cycle. Under these conditions, however, the polymerase appears to be stable. The overall data indicated that some unspecified process is responsible for the apparent instability of viral-induced RNA polymerase between 4 and 6 hr and that it becomes inoperative toward the end of the replicative cycle.

Infection of L cells with mengovirus (2, 4) and of HeLa cells with poliovirus (4) results in the appearance of an actinomycin-resistant ribonucleic acid (RNA) polymerase activity which does not appear to be present in uninfected cells. This viral-induced synthesis of RNA can also be detected in intact cells by measuring the incorporation of uridine into acid-insoluble material in the presence of actinomycin D (3, 7, 8, 9, 13). It is generally assumed that the rate of uridine incorporation in the presence of actinomycin D by infected cells is an index of RNA polymerase activity, but quantitative studies regarding this relationship are lacking. In this communication, the general characteristics of an RNA polymerase from mengovirus-infected Novikoff hepatoma cells will be described. Evidence will be presented that the rate of uridine incorporation by infected cells is not a true index of RNA polymerase activity. The results of studies of the time course of formation of mature virus and of viral RNA polymerase and the effect of puromycin on its activity will also be presented.

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MATERIALS AND METHODS

Materials. Puromycin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. All other materials were the same as described by Plagemann and Swim (9).

Cell culture and viral infection. The cultivation of Novikoff hepatoma cells, strain N1S1-67, in vitro and their infection with mengovirus have been described previously (9).

Cell fractionation. Cells were disrupted in 20 volumes of a solution (B1) containing 0.01 M tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.4), 0.01 M KCl, and 0.0015 M MgCl_2 , as described previously (9). The nuclei were sedimented at $800 \times g$ for 10 min, and the postnuclear fraction was centrifuged at $105,000 \times g$ for 1 hr. The resulting pellet containing the mitochondria and microsomes (MM fraction) was suspended in B1 with a Douce homogenizer to yield approximately 5 mg of protein per ml.

Enzyme assays. The viral-induced RNA polymerase was assayed as follows. Samples of 0.2 to 0.4 ml of cell material containing 0.6 to 1.5 mg of protein were mixed with 0.4 ml of reaction mixture containing (in micromoles): magnesium acetate, 5; Tris-HCl (pH 8.2), 50; adenosine triphosphate (ATP), cytidine triphosphate (CTP), and uridine triphosphate (UTP), 0.06 each; sodium phosphoenol pyruvate (PEP), 4; and 8 μg of pyruvate kinase, 10 μg of actinomycin D, and 0.1 μC of guanosine triphosphate (GTP) C^{14} (13 $\mu\text{C}/\mu\text{mole}$) or 1 μC GTP- H^3 (1,700 $\mu\text{C}/\mu\text{mole}$). The mix-

ture was incubated at 37 C for 30 min unless otherwise specified, and then was analyzed for acid-insoluble radioactivity as described by Plagemann and Swim (9). Whole cells were lysed prior to assay by suspending approximately 10^7 cells in 0.4 ml of B1 and 0.02 ml of 10% sodium deoxycholate (DOC) at 0 C.

Conditions for the assay of the deoxyribonucleic acid (DNA)-dependent RNA polymerase of Novikoff cells are specified under Results.

Adenosine triphosphatase activity was estimated according to the method of Ernster and Jones (5).

Additional methods. The incorporation of labeled uridine into RNA by intact cells and chemical analysis for protein, RNA, and DNA have been described (9). Suspensions of cells labeled with uridine were also treated with 1% DOC and 20 μ g/ml of ribonuclease at 37 C for 20 min, and the acid-insoluble material which remained was referred to as DOC-ribonuclease resistant RNA (9).

RESULTS

General characteristics of the viral-induced RNA polymerase from the MM fraction. The data presented in Table 1 demonstrate that the MM fraction from Novikoff cells infected with mengo-virus incorporated about 100 times as much GTP, CTP, and UTP into an acid-insoluble product in the presence of actinomycin D as did the identical fraction from uninfected cells. The MM fraction from uninfected cells contains considerable polyadenylic acid (poly A) polymerase (Plagemann and Swim, *in preparation*), and for this reason the amount of ATP incorporated by the MM fraction from infected cells was only about twofold greater than that of uninfected cells. The RNA polymerase activity

TABLE 1. Incorporation of nucleoside triphosphate by MM fraction from infected and uninfected cells

Labeled substrate*	Amt (μ moles) incorporated†	
	Mengo-infected‡	Uninfected
GTP- C^{14}	163	1.6
CTP- C^{14}	169	2.4
UTP- C^{14}	166	1.5
ATP- C^{14}	355	150

* The complete reaction mixture of 0.6 ml contained: 5 μ moles of magnesium acetate, 50 μ moles of Tris-HCl (pH 8.2), 4 μ moles of PEP, 8 μ g of pyruvic kinase, 10 μ g of actinomycin D, 0.04 μ moles each of ATP, CTP, GTP, and UTP, and MM fraction containing 1 mg of protein. In each reaction, one nucleoside triphosphate was labeled with C^{14} (2.5 μ C/ μ mole).

† Means of duplicate determinations.

‡ The MM fraction was prepared from cells 6 hr after infection with a multiplicity of 20.

TABLE 2. Requirements of viral-induced RNA polymerase

Reaction mixture*	GTP- C^{14} incorporated (μ moles)	
Complete.....	238,	241
– ATP.....	54,	48
– CTP.....	64,	58
– UTP.....	62,	65
– ATP, CTP, UTP.....	6,	7
– Magnesium.....	12,	10
– PEP, pyruvic kinase.....	75,	75
– Magnesium + manganese, 1.5 μ moles.....	41,	33
+ Pyrophosphate, 10 μ moles.....	5,	6
+ Monophosphate, 10 μ moles.....	213,	240

* The complete reaction mixture of 0.7 ml contained: 5 μ moles of magnesium acetate, 50 μ moles of Tris-HCl (pH 8.2), 4 μ moles of PEP, 8 μ g of pyruvic kinase, 10 μ g of actinomycin D, 0.06 μ mole each of ATP, CTP, and UTP, 0.1 μ C of GTP- C^{14} (13 μ C/ μ mole), and MM fraction from cells 6 hr after infection containing 1.2 mg of protein.

of infected cells, which is resistant to actinomycin D, is hereafter referred to as the viral-induced RNA polymerase or simply viral polymerase.

The general requirements of the viral-induced RNA polymerase from an MM fraction are illustrated in Table 2. The results of other studies demonstrated that the pH optimum of the reaction is 8.2 and that 3 μ moles of Mg^{++} is required per reaction mixture for maximal activity under conditions described in the footnote to Table 2. The requirement for an ATP-generating system was explained by the observation that the MM fraction from either infected or uninfected cells degraded approximately 2 μ moles of ATP per mg of protein per hr when the system was saturated with respect to ATP.

The time course of the polymerase reaction is illustrated in Fig. 1. The reaction proceeded linearly and maximally for about 20 min, and then the rate declined sharply. In routine assays for polymerase, the mixture was incubated for 30 min, which allows the reaction to proceed practically to completion. Under these conditions, the polymerase activity of the MM fraction was directly proportional to its concentration up to about 2 mg of protein per reaction mixture.

Distinguishing the viral-induced RNA polymerase from the DNA-dependent RNA polymerase of Novikoff cells. The data presented in Table 3 demonstrate that the viral-induced RNA polymerase can be distinguished from the DNA-dependent RNA polymerase of the host cell in several ways. First, the nuclear enzyme requires

a DNA template (10, 11) and is thus inhibited by treatment with deoxyribonuclease or actinomycin D. Second, either ammonium sulfate or deoxycholate (6) markedly stimulated the activity of the DNA-dependent polymerase of the nuclear fraction. The viral-induced RNA polymerase, on the other hand, was strongly inhibited by ammonium sulfate. Third, the activity of the DNA-dependent RNA polymerase was rapidly lost upon storage at -17°C , whereas the viral-induced enzyme was relatively stable under these conditions.

Relationship between viral-induced RNA polymerase activity of cell-free extracts and uridine incorporation by intact cells. The results of other studies (9) indicated that the actinomycin-resistant incorporation of uridine by infected Novi-

TABLE 3. *Distinguishing characteristics of viral-induced RNA polymerase and nuclear RNA polymerase of Novikoff cells*

Treatment of cellular fraction prior to assay	Counts per min incorporated per reaction*	
	MM fraction	Nuclei
None	1,700	1,600
Actinomycin D, 10 μg	1,680	190
Deoxyribonuclease, 10 μg , 22 $^{\circ}\text{C}$, 10 min.....	1,560	280
$(\text{NH}_4)_2\text{SO}_4$, 15% saturation.....	180	5,000
Deoxycholate, 0.5%.....	1,050	5,400
7 days at -17°C	1,670	290

* The complete reaction mixture of 0.7 ml contained: 5 μmoles of magnesium acetate, 50 μmoles of Tris-HCl (pH 8.2), 4 μmoles of PEP, 8 μg of pyruvic kinase, 0.06 μmole each of ATP, CTP, and UTP, 0.1 μC of GTP- H^3 (1,700 $\mu\text{C}/\mu\text{mole}$), and MM fraction from cells 6 hr after infection containing 1.1 mg of protein or nuclear fraction from uninfected cells containing 1.2 mg of protein. Means of duplicate determinations.

FIG. 1. Time course of viral-induced RNA polymerase reaction. A 7-ml amount of MM fraction from cells 6 hr after infection was incubated with 14 ml of reaction mixture containing (per ml): 12.5 μmoles of magnesium acetate, 125 μmoles of Tris-HCl (pH 8.2), 10 μmoles of PEP, 20 μg of pyruvic kinase, 25 μg of actinomycin D, 0.15 μmole each of ATP, CTP, and UTP, and 2.5 μC of GTP- H^3 (1,700 $\mu\text{C}/\mu\text{mole}$). Duplicate 0.6-ml samples were removed at intervals and assayed for acid-insoluble radioactivity. At 30 min, two 3-ml samples were removed: one received 1 ml of additional MM fraction and the other, 2 ml of additional reaction mixture. Duplicate 0.8-ml and 1-ml samples, respectively, were assayed for acid-insoluble radioactivity.

koff cells reflects the formation of viral-induced RNA polymerase in the cell. This was confirmed by the data presented in Fig. 2, which demonstrate that actinomycin-resistant synthesis of RNA by intact cells began with the appearance of viral-induced RNA polymerase activity as measured in cell-free extracts. It will be noted, however, that the rate of uridine incorporation by intact cells was not a true index of enzyme activity. The failure of cells to incorporate uridine near the end of the replicative cycle, when polymerase activity was maximal, probably resulted from loss of ability to synthesize or to retain nucleoside triphosphates as a consequence of cell damage. A biphasic formation of viral RNA polymerase similar to that illustrated in Fig. 2 was observed when the concentration of GTP in the reaction mixture was increased 100-fold. It is therefore considered unlikely that the secondary rise in enzyme activity reflected a decrease in the intracellular pool of GTP.

Other experiments showed that the biphasic curve for the production of polymerase was not due to the sequential production and destruction of an inhibitor. Samples of postnuclear fractions obtained from cells 4 and 7 hr after infection were tested singly and in various combinations for polymerase activity. In all instances, the activity of the combined postnuclear fractions was equivalent to the sum of the individual fractions. Several preparations of RNA were extracted with hot phenol-sodium dodecylsulfate from infected cells

FIG. 2. Time course of formation of RNA polymerase in relation to uridine incorporation by intact cells in presence of actinomycin D. A suspension of cells at 2×10^6 per milliliter infected with a multiplicity of 30 was divided into two parts. Cells from 5-ml samples were assayed for RNA polymerase activity. The second part of the suspension received 2 $\mu\text{g}/\text{ml}$ of actinomycin D and 0.5 $\mu\text{mole}/\text{ml}$ of 5-fluorodeoxyuridine at 1.5 hr after infection and 0.01 $\mu\text{mole}/\text{ml}$ of uridine- H^3 (100 $\mu\text{C}/\mu\text{mole}$) at 2 hr. Samples (1-ml) were assayed in duplicate for total acid-insoluble radioactivity and that resistant to treatment with DOC-ribonuclease.

containing high levels of polymerase. None of these stimulated the viral-induced RNA polymerase activity of either the MM fraction or whole cells lysed with DOC obtained at intervals between 3 and 7 hr after infection.

Temporal relationship between polymerase activity and the production of mature virus. The production of mature virus can be estimated by measuring the incorporation of labeled uridine by whole cells into RNA which is resistant to ribonuclease in the presence of DOC (9). The data in Fig. 2 demonstrate that polymerase activity remained constant or decreased slightly during the period of maximal production of virus. It will also be noted that the secondary rise in polymerase occurred when the rate of production of virus began to decline and that it continued to increase after viral maturation had ceased.

Effect of puromycin on formation of viral-induced RNA polymerase. The experiment outlined in Fig. 3 was designed to assess the role of protein synthesis in the secondary rise in polymerase at the end of the replicative cycle. There was no further increase in polymerase activity when puromycin was added at 6.7 hr after infection, which suggested that de novo synthesis of enzyme is required for the secondary rise in activity.

Effect of temperature on time course of viral-induced RNA synthesis. In an earlier report (9), variations in the length of the replicative cycle of mengovirus in Novikoff cells were noted. Temperature was considered to be a potential factor, and consequently its effect on the time course of viral-induced RNA synthesis was investigated. The results of one of these experiments are presented in Fig. 4. It will be noted that lowering the temperature from 37 to 35 C caused a delay in the production of RNA polymerase, and also in the incorporation of uridine by intact cells in the presence of actinomycin D, by approximately 1 hr without significantly affecting either of these parameters quantitatively. The data, therefore, suggest that some step(s) prior to the production of polymerase was extremely temperature-dependent.

FIG. 3. Effect of puromycin on the formation of viral-induced RNA polymerase. Samples of a suspension of infected cells (2×10^6 cells) per milliliter received 100 $\mu\text{g}/\text{ml}$ of puromycin at indicated times. Cells from 2.5-ml samples were assayed in duplicate for RNA polymerase activity. Incorporation of uridine- H^3 into DOC-ribonuclease-resistant RNA was determined as described in the legend to Fig. 2.

FIG. 4. Effect of temperature on the formation of viral-induced RNA polymerase. A suspension of cells at 10 cells per milliliter was infected with a multiplicity of 25, and after 20 min at 37 C was divided into two portions. One sample was diluted fivefold with basal medium 42 (BM42) prewarmed at 35 C, and the other with BM42 prewarmed at 37 C. The suspensions were then incubated on a rotary shaker at 35 and 37 C, respectively. (A) Cells from 2.5-ml samples were assayed in duplicate for RNA polymerase activity. (B) A portion of each suspension received 1 μ g/ml of actinomycin D, 0.5 μ mole/ml of FUDR, and 0.01 μ mole/ml of uridine- H^3 (100 μ c/ μ mole) at indicated times. Cells from 1-ml samples were assayed in duplicate for total acid-insoluble radioactivity.

DISCUSSION

The results reported here confirm the findings of Baltimore and Franklin (2, 4), in that an RNA polymerase activity which is resistant to actinomycin D develops in mammalian cells after infection with mengovirus (Table 1). This is referred to as the viral-induced RNA polymerase, and it is readily distinguished from the DNA-dependent RNA polymerase of Novikoff cells (Table 3). The viral-induced polymerase is probably a new protein, but the possibility that it might reflect increased activity of a normal enzyme has not been rigorously excluded. The requirements for optimal activity of the viral polymerase (Table 2) are similar to those described for other systems (2, 4, 12).

The initiation of actinomycin-resistant synthesis of RNA by infected cells as measured by the incorporation of uridine into an acid-insoluble product coincides with the appearance of viral-induced RNA polymerase (Fig. 2). The rate of RNA synthesis as measured by uridine incorporation in the presence of actinomycin D, however, is not a true index of the polymerase activity as determined in cell-free extracts. For example, the rate of uridine incorporation remains constant between 3 and 4 hr while the

polymerase continues to increase. Incorporation of uridine into RNA is, therefore, limited by factors other than the polymerase activity.

The time course of the formation of the viral-induced polymerase (Fig. 2) could be interpreted as simply reflecting some type of metabolic control of the polymerase at various stages of the replicative cycle. Polymerase activity should be dependent on both the amount of enzyme protein and the degree of saturation with respect to template. Failure of the RNA extracted from infected cells to alter the activity of the polymerase isolated at various times from 3 to 7 hr implies that the observed changes in level of enzyme (Fig. 2 and 3) are not due to variations in the availability of template. Part of the data could be explained if the polymerase is incorporated into mature virus and thereby rendered inactive. For example, the results in Fig. 2 would indicate that production and utilization of enzyme are occurring at approximately the same rate between 4 and 6 hr, since the level of activity remains relatively constant during this period. Similarly, if synthesis of enzyme proceeds after viral maturation ceases, the observed secondary rise in polymerase (Fig. 2) would be anticipated. The polymerase activity would be

expected to increase between 3 and 4 hr, because little mature virus is made during this period (Fig. 2; 9). Attempts to verify the foregoing hypothesis by demonstrating RNA polymerase activity in purified virus have been unsuccessful.

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LITERATURE CITED

1. BALTIMORE, D., H. J. EGGERS, R. M. FRANKLIN, AND I. TAMM. 1963. Poliovirus-induced RNA polymerase and the effects of virus-specific inhibitors on its production. *Proc. Natl. Acad. Sci. U.S.* **49**:843-849.
2. BALTIMORE, D., AND R. M. FRANKLIN. 1962. Preliminary data on a virus-specific enzyme system responsible for the synthesis of viral RNA. *Biochem. Biophys. Res. Commun.* **9**:388-392.
3. BALTIMORE, D., AND R. M. FRANKLIN. 1962. The effect of Mengovirus infection on the activity of the DNA-dependent RNA polymerase of L-cells. *Proc. Natl. Acad. Sci. U.S.* **48**:1383-1390.
4. BALTIMORE, D., AND R. M. FRANKLIN. 1963. A new ribonucleic acid polymerase appearing after Mengovirus infection of L-cells. *J. Biol. Chem.* **238**:3395-3400.
5. ERNSTER, L., AND L. C. JONES. 1962. A study of the nucleoside tri- and diphosphate activities of rat liver microsomes. *J. Cell Biol.* **15**:563-578.
6. GOLDBERG, I. H. 1961. Ribonucleic acid synthesis in nuclear extracts of mammalian cells grown in suspension culture; effect of ionic strength and surface-active agents. *Biochim. Biophys. Acta* **51**:201-204.
7. HAUSEN, P., AND D. W. VERWOERD. 1963. Studies on the multiplication of a member of the Columbia SK group (Me virus) in L cells. III. Alteration of RNA and protein synthetic patterns in virus-infected cells. *Virology* **21**:617-627.
8. HOMMA, M., AND A. F. GRAHAM. 1963. Synthesis of RNA in L cells infected with Mengo virus. *J. Cellular Comp. Physiol.* **62**:179-192.
9. PLAGEMANN, P. G. W., AND H. E. SWIM. 1966. Replication of mengovirus. I. Effect on synthesis of macromolecules by host cell. *J. Bacteriol.* **91**:2317-2326.
10. WEISS, S. B. 1960. Enzymatic incorporation of ribonucleoside triphosphates into the interpolynucleotide linkages of ribonucleic acid. *Proc. Natl. Acad. Sci. U.S.* **46**:1020-1030.
11. WEISS, S. B. 1962. Biosynthesis of ribopolynucleotides. *Federation Proc.* **21**:120-126.
12. WEISSMANN, C., L. SIMON, AND S. OCHOA. 1963. Induction by an RNA phage of an enzyme catalyzing incorporation of ribonucleotides into ribonucleic acid. *Proc. Natl. Acad. Sci. U.S.* **49**:407-414.
13. ZIMMERMAN, E. F., M. HEETER, AND J. E. DARNELL. 1963. RNA synthesis in poliovirus-infected cells. *Virology* **19**:400-408.