A New Ribonucleic Acid Polymerase Appearing after Mengovirus Infection of L-Cells*

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The ribonucleic acid viruses represent an anomaly in the biological world, being its only known entities which lack deoxyribonucleic acid. Nevertheless, these viruses readily transmit genetic characters, they can be mutated by agents which affect DNA (1), and their RNA is sufficient to determine their heritable characteristics (2). In the light of present knowledge of genetic mechanisms, especially as elucidated by the work of Jacob and Monod (3), it is generally believed that viral RNA can function as a type of messenger RNA and experimental support for this notion exists (4). This leaves open the question of how RNA can duplicate, since cellular messenger RNA does not appear to be directly replicated, but is a complementary copy of DNA. The work described here was undertaken in an attempt to clarify the mechanism of viral RNA duplication.

Simon (5), Reich and Franklin (6), and Reich et al. (7) have demonstrated that metabolic inhibitors which prevent DNA-mediated DNA and RNA synthesis have no effect on the multiplication of small RNA animal viruses (8). Further, viral RNA synthesis appears to take place in the particulate fraction of the cytoplasm of infected cells rather than in the nucleus (8). Hence it seemed likely that viral RNA is directly duplicated in a reaction mediated by an RNA-dependent RNA polymerase. Such an enzymatic activity has been demonstrated in infected cells and, although it has not been directly shown that it is RNA-dependent, its characterization is complete enough to warrant consideration at this time. Weissman, Simon, and Ochoa (9) and Kaye, Ortiz, and August (10) have reported a similar activity in bacteria infected with an RNA bacteriophage. A preliminary report of this work has been published (11).

EXPERIMENTAL PROCEDURE

Preparation of Infected Cells—L-Cells (strain 929) were maintained as monolayer cultures and used as suspension cultures. Growth in suspension in Eagle's spinner medium plus nonessential amino acids and 7% fetal calf or newborn calf serum has been described, as has the method of preparation of infected cultures (12).

Enzyme Assay—The following assay has been found to give optimal results and was used throughout with minor modifications as indicated. The reaction mixture (0.5 ml) contained 20 μg of phosphoenolpyruvate kinase, 5 μmoles of phosphoenolpyruvate, 30 μmoles of Tris-HCl buffer, pH 8.1, 5 μmoles of magnesium acetate, 1 μg of actinomycin, and ATP, UTP, GTP, and CTP, one of which was labeled with C14. To each tube was added 0.2 ml of an enzyme preparation (0.5 to 2 mg of protein). After incubation at 35°C, usually for 10 minutes, the mixtures were rapidly chilled and 0.5 ml of 0.1 M sodium pyrophosphate was added (13), followed by 5 ml of 0.5 M perchloric acid. After 10 minutes in ice, the suspension was centrifuged and the precipitate was washed three times with perchloric acid and once with ethanol ether (1:1) by centrifugation and resuspension. The acid-insoluble material was dissolved with 1 ml of concentrated formic acid, transferred to metal planchets, dried, and counted in a windowless gas flow counter. In some experiments, samples which were unincubated gave high levels of radioactivity (about 200 c.p.m.); this value could be reduced to less than 15 c.p.m. by the following procedure, which did not affect the enzymic incorporation of nucleotides. After the initial acid precipitation, 0.3 ml of 0.5 M NaOH was added, followed rapidly by 0.5 ml of 0.1 M sodium pyrophosphate and 4 ml of 0.5 M perchloric acid. After 10 minutes, the suspension was centrifuged and the precipitate was washed twice more with perchloric acid and once in ethanol ether, dissolved in formic acid, and counted as before. All radioactivity measurements were corrected by subtraction of the counting rate of an unincubated control sample, which never exceeded 10% of incubated samples. No correction for self-absorption was made.

Electrophoresis Experiments—Reaction conditions were identical with those used for C14 incorporation studies except for the use of α-32P-ribonucleoside triphosphates (about 10,000 c.p.m. per pmole) instead of the C14-ribonucleoside triphosphates. After the ethanol-ether wash, the precipitate was hydrolyzed for 18 hours at 37°C in 0.3 ml of 0.3 N KOH. The resulting solution was acidified to pH 1 with 10 N perchloric acid and centrifuged, and the insoluble proteins and potassium perchlorate were discarded. The supernatant solution was taken to pH 3.5 with 10 N KOH, clarified by centrifugation, and dried overnight at 37°C under a continuous air flow. The residue was taken up in 10 to 20 μl of buffer and applied to Whatman No. 3MM paper, and electrophoresis was carried out in 0.1 M ammonium formate buffer, pH 3.5, for 1/2 hours at 35 volts per cm (14). The nucleotides were detected by ultraviolet absorption (the ribosomal RNA in the enzyme preparation supplying the bulk of the optical density) and autoradiography on x-ray film. There was exact correspondence of optical density and radioactivity; parallel marker spots for the 2'(3')-mononucleotides allowed classification of the spots as AMP, CMP, UMP, and GMP. The ultraviolet-absorbing regions were cut out and counted directly in a windowless gas flow counter.

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flow counter; the results are expressed as percentage of total radioactivity.

Materials—C14-Nucleoside triphosphates were purchased from Schwarz BioResearch, Inc. Unlabeled nucleoside triphosphates, pyruvate kinase, phosphoenolpyruvate, and myokinase were obtained from Sigma Chemical Company. RNase and DNase were products of Worthington Biochemical Corporation.

UTP271 was a gift from Dr. J. Hurwitz. Dr. T. August kindly provided GTP22 and P32-labeled cyanoethylphosphate. AMP21 was prepared by the method of Tener (15) and converted to ATP22 with myokinase, phosphoenolpyruvate, and pyruvate kinase (16).1

RESULTS

Enzyme Preparation

Two methods were used for preparation of cell fractions which incorporate ribonucleotides into an acid-insoluble form. Cells infected for 5 hours were homogenized either in 0.25 M sucrose plus 10-4 M MgCl2 (sucrose-Mg), or in demineralized water followed by a treatment with salt at high concentration. The former method, which causes little nuclear breakage, was used in early work on this system and the latter, which yields more active preparations, has been used for later studies. Both methods are described below.

Sucrose Homogenization—Procedures described previously (12) for the homogenization of cells in a VirTis homogenizer were used except that mercaptoethanol was eliminated. Less than 5% nuclear breakage occurred as determined by measurements of DNA liberated into cytoplasmic fractions (8). After nuclei were removed by centrifugation, the cytoplasmic preparations were either used directly or fractionated further by differential centrifugation (8). For assay, centrifuged fractions were resuspended in sucrose-Mg at 5 to 10 mg of protein per ml, with an all-glass homogenizer when necessary.

Water Homogenization—Infected cells were centrifuged in the cold at 200 x g, resuspended in sucrose-Mg, recentrifuged, and either frozen at -20° or used directly. All further procedures were carried out at 0-4°. The cell pellet was suspended in 20 to 40 volumes of cold water and homogenized with 5 to 10 strokes of the tightly fitting pestle of a Dounce homogenizer (Kontes, 40-ml size). Such preparations were routinely examined by phase contrast microscopy, and the homogenization was terminated when the preparation consisted almost entirely of cell debris and nuclei without cytoplasmic tabs. If 10 homogenization strokes were not sufficient, more water was added and a few more strokes applied. The disrupted suspension was then adjusted to a final concentration of 0.1 M Tris-HCl buffer, pH 7.6, and 5 x 10-3 M magnesium chloride. One-tenth volume of 5 M NaCl was added and the preparation was mixed by rapid pipetting. After 5 to 10 minutes at 0°, the viscous suspension was diluted 2-fold with water to reduce its viscosity; this resulted in fine aggregates of nucleoprotein. After centrifugation at 800 x g for 10 minutes to remove nuclei, whole cells, and the aggregated nucleoprotein, the supernatant solution was centrifuged at 78,000 x g in a Spinco model L ultracentrifuge for 1 1/2 to 2 hours. The resulting supernatant solution was discarded, the surface of the intact pellet and the tube were rinsed once with sucrose-Mg, and the pellet was resuspended in sucrose-Mg with an all-glass homogenizer to give a suspension containing 2.5 to 10 mg of protein per ml. Such suspensions incorporate, in 10 minutes, about 0.4 mkmole of GMP-C14 per mg of protein, as opposed to the sucrose homogenates, which incorporate about 0.1 mkmole under optimal conditions. These particulate preparations from the cytoplasm of infected cells will be referred to as an "enzyme preparation" and could be stored at -20° with retention of 75% of activity after 1 week.

The incorporation of ribonucleotides catalyzed by the enzyme proceeds linearly for approximately 10 minutes. Between 15 and 30 minutes the reaction rate falls off, and the reaction ceases by 60 minutes. In one experiment the incorporation of GMP-C14 into an acid-insoluble form in 5, 10, 15, and 20 minutes was 0.11, 0.19, 0.24, and 0.31 mkmole, respectively. The rate of the reaction is proportional to enzyme concentration in the range of 2 to 0.5 mg of protein per 0.5 ml of reaction mixture. In a typical experiment the addition of 1.8, 1.2, and 0.6 mg of protein resulted in the incorporation into an acid-insoluble form of 0.28, 0.15, and 0.07 mkmole of GMP-C14, respectively, in 10 minutes.

Viral RNA Polymerase Activity and Its Properties

The microsomal fraction from infected cells incorporates about 10 times more of each of the four nucleotides (AMP, GMP, CMP, and UMP) than a similar extract from uninfected cells (Table I). Incorporation of each nucleoside triphosphate is at least partially dependent on the presence of the others. For maximal GMP incorporation, the presence of all four nucleoside triphosphates is essential (Table II). The omission of a single nucleoside triphosphate markedly reduced GMP incorporation; similar results have been obtained with ATP-C14. The ribonucleotide-incorporating activity is insensitive to actinomycin, DNase, and puromycin and is dependent on the presence of an ATP-generating system, and corresponding deoxynucleoside triphosphates do not replace their ribonucleoside counterparts (Table III). The reaction is not affected by the addition of up to 0.4 M NaCl or is sensitive to 0.02 M fluoride or 4 mM phosphate. All of the incorporated radioactivity can be sedimented

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>Infected</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP-C14, complete</td>
<td>120</td>
<td>13.8</td>
</tr>
<tr>
<td>Minus UTP and GTP</td>
<td>51.5</td>
<td>20.3</td>
</tr>
<tr>
<td>GTP-C14, complete</td>
<td>55</td>
<td>5.9</td>
</tr>
<tr>
<td>Minus CTP and ATP</td>
<td>7.8</td>
<td>7.5</td>
</tr>
<tr>
<td>UTP-C14, complete</td>
<td>50.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Minus ATP, GTP, and CTP</td>
<td>14.1</td>
<td>3.9</td>
</tr>
<tr>
<td>CTP-C14, complete</td>
<td>73.5</td>
<td>20.8</td>
</tr>
<tr>
<td>Minus ATP, CTP, and UTP</td>
<td>27.9</td>
<td>19.0</td>
</tr>
</tbody>
</table>

1 We are indebted to Dr. T. August for assistance in this preparation.
TABLE II

Effect of omission of single triphosphates on GMP-C\textsuperscript{4} incorporation

The complete reaction mixture contained 73 mpmoles of GTP-C\textsuperscript{4} (2100 c.p.m. per mpmole) and 60 mpmoles of each of the other ribonucleotides and was incubated for 15 minutes.

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>GMP-C\textsuperscript{4} incorporation c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>414</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>159</td>
</tr>
<tr>
<td>Minus CTP</td>
<td>172</td>
</tr>
<tr>
<td>Minus UTP</td>
<td>74</td>
</tr>
<tr>
<td>Minus CTP and UTP</td>
<td>65</td>
</tr>
</tbody>
</table>

TABLE III

Properties of GMP-C\textsuperscript{4} incorporation by viral RNA polymerase

Experiments involved different batches of enzyme incubated with the following amounts of GTP-C\textsuperscript{4} (in million cpm): Experiment 1 and 3, 73 (2100 c.p.m. per mpmole); Experiment 2, 53 (3380 c.p.m. per mpmole); and Experiment 4, 32 (5080 c.p.m. per mpmole). The concentration of unlabeled nucleotides was adjusted to be approximately equal to that of the GTP-C\textsuperscript{4}. For Experiment 2 both samples were first incubated at 37° for 5 minutes with either DNase or water and 0.003 M MgCl\textsubscript{2} before addition of the components of the reaction mixture.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Reaction system</th>
<th>GMP-C\textsuperscript{4} Incorporation c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Complete</td>
<td>2240</td>
</tr>
<tr>
<td></td>
<td>Plus 10 (\mu)g of actinomycin</td>
<td>2310</td>
</tr>
<tr>
<td>2</td>
<td>Complete</td>
<td>532</td>
</tr>
<tr>
<td></td>
<td>Plus 50 (\mu)g of DNase</td>
<td>526</td>
</tr>
<tr>
<td>3</td>
<td>Complete</td>
<td>761</td>
</tr>
<tr>
<td></td>
<td>Minus phosphoenolpyruvate and pyruvate kinase</td>
<td>352</td>
</tr>
<tr>
<td>4</td>
<td>Complete</td>
<td>1146</td>
</tr>
<tr>
<td></td>
<td>Minus UTP and CTP</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Minus UTP and CTP, plus TTP and dCTP</td>
<td>88</td>
</tr>
</tbody>
</table>

in 1 hour at 105,000 \(\times\) g, suggesting that the radioactivity is associated with a relatively large particle.

\(pH\) and Ionic Requirements

Incorporation is maximal between \(pH\) 7.8 and 9.5 and falls to 50% at \(pH\) 7.2. The reaction is completely dependent on added magnesium ions and shows an optimum at about 2 \text{mM} (Fig. 1); there is no inhibition by magnesium until the concentration reaches greater than 50 \text{mM}. Manganese ions markedly inhibit the reaction in the presence of optimal magnesium (Fig. 2). Even 0.2 \text{mM} manganese inhibits the reaction by 40% and, at equimolar concentrations of manganese and magnesium ions, over 90% inhibition occurs.

Nature of Product

The acid-insoluble radioactivity is completely solubilized by incubation with 0.3 M NaOH at 37° for 18 hours. When GTP-C\textsuperscript{4} or ATP-C\textsuperscript{4} was used as precursor and the alkaline hydrolysate of RNA was fractionated by electrophoresis, all of the radioactivity originally in ATP was recovered in the AMP region of the paper, whereas when C\textsuperscript{4}-GMP was incorporated, after alkaline degradation only 3% of the counts were recovered in guanosine, the rest being in GMP (Table IV). Since the guanosine spot was not entirely separated from the origin under these conditions, the few counts in the spot could be material which was not completely hydrolyzed. These results support the conclusion that essentially all of the incorporated radioactivity is in internal phosphodiester linkages rather than in terminal positions.

When guanosine-P\textsuperscript{32}-PP, adenosine-P\textsuperscript{32}-PP, or uridine-P\textsuperscript{32}-PP (CTP not tested) was incubated with an enzyme preparation and the product was hydrolyzed in base and subjected to electrophoresis, radioactivity was found in all four mononucleotides (Table V). Furthermore, the nearest neighbor frequencies to AMP so derived are little affected by a 2.5-fold increase in the amount of the unlabeled triphosphates, suggesting that these nearest neighbor frequencies are not determined by the relative concentrations of the nucleotide precursors. Similar results were found with RNA labeled with GMP\textsuperscript{32}.

With washed microsomal preparations from uninfected cells, incorporation of GMP is almost negligible although ATP shows significant incorporation. This difference between ATP and GTP is also observed with washed enzyme preparations; signifi-
Fig. 2. The effect of added Mn++. To a reaction mixture as described in Table II, with 1.8 mmoles of magnesium, were added various amounts of manganese chloride. Closed (•) and open (O) circles refer to separate experiments.

Table IV
Distribution of radioactivity in alkaline hydrolysate of C14-labeled viral RNA polymerase product

Reaction mixtures containing either 169 mmoles of ATP-C14 (1960 c.p.m. per m mole) plus 240 m moles of each of the other three ribonucleotides or 32 m moles of GTP-C14 (5080 c.p.m. per m mole) plus 120 m moles of each of the other three ribonucleotides were incubated for 15 minutes and prepared for electrophoresis. No other radioactivity except that which is indicated was demonstrable. Data are given in counts per minute.

<table>
<thead>
<tr>
<th>Labeled substrate</th>
<th>Distribution of radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ud</td>
</tr>
<tr>
<td>Guanosine-P14-PP</td>
<td>33.7</td>
</tr>
<tr>
<td>Uridine-P14-PP</td>
<td>36.2</td>
</tr>
<tr>
<td>Adenosine-P54-PP*</td>
<td>16.3</td>
</tr>
<tr>
<td>Adenosine-P54-PP†</td>
<td>13.8</td>
</tr>
<tr>
<td>Adenosine-P54-PP‡</td>
<td>7.7</td>
</tr>
</tbody>
</table>

* Incubated with 120 µg each of CTP, UTP, and GTP.
† Incubated with 300 µg each of CTP, UTP, and GTP.
‡ No unlabeled triphosphate was added.

Table V
Nearest neighbor analysis of viral RNA polymerase product

Experimental conditions were as described in Table IV, except that 0.1 ml of C14-labeled enzyme preparation was used. Reaction mixtures were incubated for 15 minutes and prepared for electrophoresis. The nearest neighbor of AMP under these conditions was predominantly AMP, to a lesser extent CMP, and rarely UMP or GMP (Table V, last line). Data are given in counts per minute.

<table>
<thead>
<tr>
<th>Nearest neighbor of</th>
<th>AMP</th>
<th>GMP</th>
<th>Guanosine</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP-C14</td>
<td>165</td>
<td>204</td>
<td>6</td>
<td>&lt;3</td>
</tr>
<tr>
<td>GTP-C14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effect of Ribonuclease on Reaction and Product

When 10 µg of RNase are added to the reaction mixture, a variable decrease in the amount of acid-insoluble radioactivity is evident (up to 20% of "complete") in the absence of one or all of the unlabeled triphosphates whereas GMP incorporation falls to less than 5% under these conditions. For these reasons, a preparation of enzyme incubated with α-P2-ATP in the absence of the other triphosphates was hydrolyzed in base and subjected to electrophoresis. The nearest neighbor of AMP under these conditions was predominantly AMP, to a lesser extent CMP, and rarely UMP or GMP (Table V, last line).

Table VI
Effect of ribonuclease on product of viral RNA polymerase

In Experiment 1a, a sucrose homogenization enzyme preparation was employed; in Experiment 1b and 1c, a preparation made by water homogenization. ATP-C14 and UTP-C14 incubations were carried out as in Table I, GTP-C14 incubation, as in Table III.

In Experiment 2a, a three times perchloric acid-washed, ethanol-ether-extracted sample was dried and then dispersed in 0.4 ml of 0.01 M Tris-HCl, pH 7.2, and readjusted to pH 7.2 with NaOH. Crystalline RNase (0.1 ml of a 0.2 mg per ml solution) was added, and the tube was left at 37°C for 30 minutes and then chilled. Sodium pyrophosphate (0.5 ml of 0.1 M) was added, followed by 5 ml of 0.5 M perchloric acid. The resulting precipitate was washed once with perchloric acid, once with ethanol-ether, and counted. The control sample was similarly treated except that 0.1 ml of Tris buffer replaced the RNase solution. In Experiment 2b, a 30-minute-incubated reaction mixture was directly extracted at 60°C with phenol plus 0.5% sodium dodecyl sulfate. An aliquot of the phenol extract was incubated in 0.6 ml with 0.2 M Tris, pH 7.4, and 20 µg of ribonuclease at 37°C for 15 minutes. The control was identically treated except for the absence of enzyme. After incubation, 1 mg of bovine serum albumin was added, and the samples were treated as in Experiment 2a.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Acid-insoluble radioactivity (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. RNase (10 µg) added to reaction tube</td>
<td></td>
</tr>
<tr>
<td>a. ATP-C14:</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>252</td>
</tr>
<tr>
<td>Plus RNase</td>
<td>182</td>
</tr>
<tr>
<td>b. UTP-C14:</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>1063</td>
</tr>
<tr>
<td>Plus RNase</td>
<td>623</td>
</tr>
<tr>
<td>c. GTP-C14:</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>1146</td>
</tr>
<tr>
<td>Plus RNase</td>
<td>854</td>
</tr>
<tr>
<td>2. RNase treatment of product</td>
<td></td>
</tr>
<tr>
<td>a. In perchloric acid precipitate:</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>860</td>
</tr>
<tr>
<td>Plus RNase</td>
<td>49</td>
</tr>
<tr>
<td>b. Phenol extract of reaction mixture:</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>97</td>
</tr>
<tr>
<td>Plus RNase</td>
<td>18</td>
</tr>
</tbody>
</table>
phodiester linkages, and since each of the tested nucleoside
triphosphates is incorporated next to all of the other mononucleo-
tides, the enzyme under consideration has the properties of an
RNA polymerase. Since an ATP-generating system is required
for maximal activity, it would appear that the precursors are
ribonucleoside triphosphates rather than the ribonucleoside di-
phosphates. The lack of inhibition by phosphate differentiates
the enzyme from polynucleotide phosphorylase. The enzyme
system incorporates, in *vivo*, in 15 minutes, between 10^{-4}
and 10^{4} viral equivalents of RNA per cell equivalent of enzyme
preparation. This is approximately equal to the maximal rate of virus
synthesis in infected cells. A large percentage, if not all, of the
viral RNA polymerase activity appears to be in the cytoplasm
of the infected cell, since sucrose homogenization releases much
of the enzyme while breaking few nuclei.

Neither DNA nor f2 bacteriophage RNA will stimulate incorp-
oration, and no major purification of the polymerase has yet
been achieved. However, the fact that the nearest neighbor
frequencies are unchanged by a 2.5-fold increase in the level of
unlabeled nucleoside triphosphate (see Table V) suggests that
there is a factor in the enzyme preparation which is determining
these ratios. The lack of inhibition by actinomycin or DNase
precludes DNA as a template in the reaction (17).

A number of lines of evidence indicate that this enzyme is not
present in uninfected cells. Study of actinomycin-inhibited cells
indicates that the only DNA-independent reactions occurring in
L-cells involve terminal and subterminal addition to preformed
soluble RNA molecules (18). Also, as demonstrated here,
imicrosomal extracts from uninfected L-cells show no incorpor-
oration activity of one nucleotide which is dependent on the addi-
tion of others. When α-32P-ATP was used as substrate, the in-
corporation in enzyme preparations which occurred in the absence
of the other nucleotides involved mainly polyadenylic acid
formation, as shown by the nearest neighbor studies, and some
incorporation next to CMP. The formation of polyadenylic
acid probably is catalyzed by the ribosomal enzyme isolated by
August, Ortiz, and Hurwitz (19) whereas the incorporation next
to CMP is probably terminal addition to transfer RNA species
(20).

The DNA-dependent RNA polymerase will also use RNA as a
primer (21, 22). Since much of the DNA-dependent polymerase
activity disappears after Mengovirus infection (12), it might be
imagined that this enzyme migrates into the cytoplasm after
infection. Several arguments against this hypothesis can be
raised. The nuclear enzyme is stimulated by manganese ions
but the virus polymerase is inhibited, even at very low concen-
trations. Furthermore, the decrease of nuclear activity occurs
much earlier than the increase of cytoplasmic incorporation (8).
Also, in poliovirus-infected HeLa cells a similar viral polymerase
appears 3 hours after infection (23, 24), before any decrease of
nuclear RNA synthesis is evident (25). Under two conditions
natural RNA synthesis decreases without the appearance of
virus RNA synthesis: when poliovirus-infected HeLa cells are
treated with guanidine at 2 hours after infection (26) and when
infected cells are kept at a supraoptimal temperature (27).
Lastly, there is evidence that the viral polymerase activity is
markedly unstable and that the enzyme must be constantly
renewed by a process requiring protein synthesis (28).\(^2\)

\(1\) R. M. Franklin, results to be published.
\(2\) D. Baltimore and R. M. Franklin, results to be published.

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**DISCUSSION**

Since the product of this reaction is sensitive to alkali and
RNase and contains labeled mononucleotides in internal phos-
phodiester linkages, and since each of the tested nucleoside
triphosphates is incorporated next to all of the other mononucleo-
tides, the enzyme under consideration has the properties of an
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**DISCUSSION**

Since the product of this reaction is sensitive to alkali and
RNase and contains labeled mononucleotides in internal phos-
phodiester linkages, and since each of the tested nucleoside
triphosphates is incorporated next to all of the other mononucleo-
tides, the enzyme under consideration has the properties of an
RNA polymerase. Since an ATP-generating system is required
for maximal activity, it would appear that the precursors are
ribonucleoside triphosphates rather than the ribonucleoside di-
phosphates. The lack of inhibition by phosphate differentiates
the enzyme from polynucleotide phosphorylase. The enzyme
system incorporates, in *vivo*, in 15 minutes, between 10^{-4}
and 10^{4} viral equivalents of RNA per cell equivalent of enzyme
preparation. This is approximately equal to the maximal rate of virus
synthesis in infected cells. A large percentage, if not all, of the
viral RNA polymerase activity appears to be in the cytoplasm
of the infected cell, since sucrose homogenization releases much
of the enzyme while breaking few nuclei.

Neither DNA nor f2 bacteriophage RNA will stimulate incorp-
oration, and no major purification of the polymerase has yet
been achieved. However, the fact that the nearest neighbor
frequencies are unchanged by a 2.5-fold increase in the level of
unlabeled nucleoside triphosphate (see Table V) suggests that
there is a factor in the enzyme preparation which is determining
these ratios. The lack of inhibition by actinomycin or DNase
precludes DNA as a template in the reaction (17).

A number of lines of evidence indicate that this enzyme is not
present in uninfected cells. Study of actinomycin-inhibited cells
indicates that the only DNA-independent reactions occurring in
L-cells involve terminal and subterminal addition to preformed
soluble RNA molecules (18). Also, as demonstrated here,
imicrosomal extracts from uninfected L-cells show no incorpor-
oration activity of one nucleotide which is dependent on the addi-
tion of others. When α-32P-ATP was used as substrate, the in-
corporation in enzyme preparations which occurred in the absence
of the other nucleotides involved mainly polyadenylic acid
formation, as shown by the nearest neighbor studies, and some
incorporation next to CMP. The formation of polyadenylic
acid probably is catalyzed by the ribosomal enzyme isolated by
August, Ortiz, and Hurwitz (19) whereas the incorporation next
to CMP is probably terminal addition to transfer RNA species
(20).

The DNA-dependent RNA polymerase will also use RNA as a
primer (21, 22). Since much of the DNA-dependent polymerase
activity disappears after Mengovirus infection (12), it might be
imagined that this enzyme migrates into the cytoplasm after
infection. Several arguments against this hypothesis can be
raised. The nuclear enzyme is stimulated by manganese ions
but the virus polymerase is inhibited, even at very low concen-
trations. Furthermore, the decrease of nuclear activity occurs
much earlier than the increase of cytoplasmic incorporation (8).
Also, in poliovirus-infected HeLa cells a similar viral polymerase
appears 3 hours after infection (23, 24), before any decrease of
nuclear RNA synthesis is evident (25). Under two conditions
natural RNA synthesis decreases without the appearance of
virus RNA synthesis: when poliovirus-infected HeLa cells are
treated with guanidine at 2 hours after infection (26) and when
infected cells are kept at a supraoptimal temperature (27).
Lastly, there is evidence that the viral polymerase activity is
markedly unstable and that the enzyme must be constantly
renewed by a process requiring protein synthesis (28).\(^2\)

\(1\) R. M. Franklin, results to be published.
\(2\) D. Baltimore and R. M. Franklin, results to be published.
SUMMARY

An enzyme which catalyzes the incorporation into an acid-insoluble form of the ribonucleoside monophosphates of adenine, guanine, uracil, and cytosine has been demonstrated in cytoplasmic extracts from Mengovirus-infected L-cells. Less than 1% of the maximal activity is found in similar extracts from uninfected cells. For this activity to be manifest, Mg++ plus all four ribonucleoside triphosphates are required along with an adenosine triphosphate-generating system. Neither actinomycin nor deoxyribonuclease affects the activity. The product contains mononucleotides in internal phosphodiester linkages and the four constituents are nonrandomly distributed.

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A New Ribonucleic Acid Polymerase Appearing after Mengovirus Infection of L-Cells

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