The Biosynthesis of Ribonucleic Acid following Infection with a Ribonucleic Acid Virus

M. J. Cline,* R. Eason,† and R. M. S. Smellie

From the Department of Biochemistry, The University, Glasgow, Scotland

(Received for publication, December 18, 1962)

The replication of deoxyribonucleic acid viruses is dependent on the synthesis of deoxyribonucleic acid, and increases in the activity of deoxyribonucleic acid nucleotidyltransferase (DNA polymerase) have been demonstrated in bacterial and mammalian cells infected with deoxyribonucleic acid viruses (1, 2).

In contrast, several lines of evidence suggest that synthesis of DNA is not a prerequisite of the replication of RNA viruses. Thus DNA synthesis declines in "L" cells infected with the RNA-containing Mengo virus (3) and inhibitors of DNA synthesis fail to interfere with the production of infective RNA viruses in HeLa cells (4, 5).

These observations raise the question as to whether the synthesis of viral RNA uses existing host cell DNA and therefore is independent of DNA synthesis or is primed by the infecting RNA directly. A partial solution to this problem has been provided by the demonstration that there is a decline in the activity of DNA-primed RNA nucleotidyltransferase (RNA polymerase) in the nuclei of "L" cells infected with Mengo virus (3) and that poliovirus replication continues at an undiminished rate in "L" cells in the presence of concentrations of actinomycin D sufficient to abolish DNA-dependent synthesis of RNA (6).

In this report, evidence is presented for the existence of independent RNA-primed and DNA-primed RNA nucleotidyltransferases in Krebs II ascites tumor cells. There was no difference in the activities of the DNA nucleotidyltransferase and DNA-primed RNA nucleotidyltransferase of uninfected and cells infected with the virus of mouse encephalomyocarditis (7). However, the levels of RNA-primed RNA nucleotidyltransferase activity increased in the infected cells and were significantly higher than in the controls. A preliminary report of this work has already appeared (8).

Experimental Procedure

Methods—dTMP32 and UMP32 were prepared from P32-orthophosphate by the method of Tener (9) and these nucleotides were phosphorylated by the procedure of Smith and Khorana (10) to yield dTMP32PP and UMP32PP. The specific activities of the nucleoside triphosphates so prepared were of the order of 20 × 10^6 c.p.m. per amole when assayed in a windowless gas flow counter.

The parent strain of Krebs II ascites tumor cells and encephalomyocarditis virus adapted for growth in these cells were kindly provided by Dr. F. Kingsley Sanders of the M. R. C. Virus Research Unit, Carshalton, England; the conditions for the preparation of the tumor, the growth of the encephalomyocarditis virus, and virus assay by hemagglutination, plaque assay, and cell death were essentially those described by Martin et al. (7).

DNA was prepared from Landeschutz mouse ascites tumor cells by the procedure of Kay, Simmons, and Dounce (11) and yeast RNA was purchased from British Drug Houses Ltd. dATP, dGTP, dCTP, ATP, GTP, and CTP were purchased from Pabst Laboratories, Sigma Chemical Company, or British Drug Houses Ltd. Carrier-free P32-orthophosphate and P32-cyanoethylphosphate were obtained from the Radiochemical Centre, Amersham, England. Crystalline pancreatic RNase and DNase were purchased from the Sigma Chemical Company.

Preparation of Enzyme—Krebs II cells were washed in phosphate-buffered NaCl, pH 7.5 (7), and suspended in Earle's medium in duplicate stoppered flasks at a concentration of 1 × 10^7 cells per ml. To one flask a preparation of encephalomyocarditis virus (3 plaque-forming units per cell) was added and the flasks were incubated in a shaking water bath at 37°. Samples of infected and uninfected were withdrawn at zero time and at one or two hourly intervals after infection and centrifuged at 750 × g for 5 minutes at 0°. The packed cells were resuspended in 0.01 M Tris buffer, pH 7.5, homogenized in a Potter-type homogenizer, and submitted to sonic vibration (50 W; 20 kc) for 45 to 60 seconds. Complete disruption of cells and nuclei by this treatment was confirmed microscopically. The suspension was centrifuged at 10,000 × g for 15 minutes at 0°, the supernatant fraction was removed, and its protein concentration adjusted to 1.5 to 2.0 mg per ml with 0.01 M Tris buffer, pH 7.5.

Nuclear and cytoplasmic fractions from infected and uninfected cells were isolated in Tween 80 by the method of Fisher and Harris (12) and enzyme preparations were obtained from the nuclei in a manner identical to that used for the whole cells. The cytoplasmic enzyme was the supernatant fluid obtained by centrifuging the cytoplasmic fraction at 10,000 × g for 15 minutes at 0°.

Enzyme Assay—DNA nucleotidyltransferase was assayed as previously described (13), DNA-primed RNA nucleotidyltransferase was assayed by a modification of the method of Weiss and Nakamoto (14), and RNA-primed RNA nucleotidyltransferase by a modification of the method of Burdon and Smellie (15).

Duplicate incubations for each assay were shaken for 45 minutes at 37° after which the reactions were terminated by quick freezing. Samples, 0.05 ml, of the reaction mixtures were pipetted onto 2.5-cm disks of Whatman No. 1 filter paper. These were washed four times with ice-cold 5% trichloroacetic acid,
twice with ethanol-ether (3:1), dried, and counted in a Nuclear-Chicago windowless gas flow detector.

Protein was estimated by the method of Lowry et al. (16).

RESULTS

DNA Nucleotidyltransferase—The incorporation of TMP\textsuperscript{32} residues from TMP\textsuperscript{32}PP into polydeoxyribonucleotides was found to be related linearly to the protein concentration in the range 0.4 to 2.0 mg per ml. Measurements of the enzyme activity were carried out within this range and the activity was expressed in micromicromoles of TMP\textsuperscript{32} incorporated per mg of protein. Defined in this way, the activity of the nuclear enzyme was about 50% of that of the cytoplasmic enzyme which in turn was comparable in activity to preparations from whole cells.

No significant differences in the activities of DNA nucleotidyltransferase from infected and uninfected cells were observed at intervals up to 5 hours after infection (Fig. 1). In four out of six experiments, no significant differences were found up to 9 hours after infection whereas in two experiments decreases of 35 to 40% in the activity of the transferase occurred between 6 and 9 hours after infection.

Similar results were obtained with enzyme fractions prepared from isolated nuclei and from the cytoplasm.

DNA-dependent and RNA-dependent RNA Nucleotidyltransferases in Normal Krebs II Cells—The incorporation of UMP\textsuperscript{32} from UMP\textsuperscript{32}PP into a form insoluble in 5% trichloroacetic acid and labile in alkali was catalyzed by the enzyme preparations and was promoted by the addition of RNA or DNA. This indicated the probable existence of both a DNA-dependent and an RNA-dependent RNA nucleotidyltransferase and these two enzymes could be distinguished by their different pH optima and ionic requirements. As can be seen from Table I, the optimal pH of the DNA-dependent system is close to 7.5 whereas that for the RNA-dependent system is near 9.5. In the DNA-primed system at pH 7.5, the reaction was greatly enhanced by the addition of MnCl\textsubscript{2} plus 2-mercaptoethanol whereas the addition of enzyme (2 mg of protein per ml) in a total volume of 0.4 ml. Where indicated 1.0 pmole each of MnCl\textsubscript{2} and 2-mercaptoethanol was included. Incubations were carried out at 37° for 45 minutes.

![Fig. 1. DNA nucleotidyltransferase activities in extracts of infected and uninfected Krebs II ascites tumor cells. The reaction mixture contained 10 μmoles of Tris buffer, pH 7.5; 9 μmoles of KCl; 0.08 μmole of EDTA; 0.5 μmole of MgCl\textsubscript{2}/K\textsubscript{2}HPO\textsubscript{4} buffer, pH 7.5; 1 μmole of MgCl\textsubscript{2}; 50 μg of DNA or RNA; and 0.2 ml of enzyme (2 mg of protein per ml) in a total volume of 0.4 ml. Where indicated 1.0 μmole each of MnCl\textsubscript{2} and 2-mercaptoethanol was included. Incubations were carried out at 37° for 45 minutes.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Primer</th>
<th>pH</th>
<th>MnCl\textsubscript{2} + 2-mercaptoethanol</th>
<th>UMP\textsuperscript{32} incorporated μmoles/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>7.5</td>
<td>+</td>
<td>220</td>
</tr>
<tr>
<td>DNA</td>
<td>7.5</td>
<td>-</td>
<td>55</td>
</tr>
<tr>
<td>DNA</td>
<td>9.5</td>
<td>+</td>
<td>75</td>
</tr>
<tr>
<td>DNA</td>
<td>9.5</td>
<td>-</td>
<td>88</td>
</tr>
<tr>
<td>Nil</td>
<td>7.5</td>
<td>+</td>
<td>42</td>
</tr>
<tr>
<td>Nil</td>
<td>9.5</td>
<td>-</td>
<td>39</td>
</tr>
<tr>
<td>RNA</td>
<td>9.5</td>
<td>+</td>
<td>223</td>
</tr>
<tr>
<td>RNA</td>
<td>7.5</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>RNA</td>
<td>7.5</td>
<td>-</td>
<td>83</td>
</tr>
</tbody>
</table>

The mixture was centrifuged at 0° for 10 minutes at 750 × g. The supernatant fluid was then assayed for DNA-dependent and RNA-dependent RNA nucleotidyltransferase activities in the absence of added primers. The results of the experiment with RNase (Fig. 2) demonstrate that at concentrations of RNase greater than 0.1 μg per ml the RNA-primed RNA nucleotidyltransferase was substantially inhibited whereas the DNA-primed RNA nucleotidyltransferase was only slightly inhibited at concentrations of RNase exceeding 1.0 μg per ml. No inhibition of the RNA-primed transferase system was observed at concentra-
tions of DNase up to 200 μg per ml although about 20% inhibition of the DNA-primed transferase was found at this concentration of DNase.

**DNA-dependent RNA Nucleotidyltransferase in Infected and Uninfected Cells**—Comparison of the enzyme preparations from infected and uninfected cells showed no significant differences in the DNA-primed RNA nucleotidyltransferase activity at intervals up to 9 hours after infection (Fig. 3). In the absence of added DNA, the incorporation of UMP\(^{32}\) varied between 10% and 48% (average of six experiments, 27%) of that observed in the DNA-primed reaction. There was no significant difference in the unprimed transferase activity of infected and uninfected cells.

**RNA-dependent RNA Nucleotidyltransferase in Injected and Uninfected Cells**—The RNA-primed RNA nucleotidyltransferase activity of the infected Krebs II cells was found to be consistently elevated by comparison with that of the uninfected cells.
FIG. 5. RNA-primed RNA nucleotidyltransferase activity of infected and uninfected Krebs II ascites tumor cells. (a) Whole cells; (b) nuclei. The assay conditions were as described in Fig. 2 with the addition of 50 μg of RNA per assay.

cells (Figs. 4 and 5). These figures present the results of a number of independent experiments in which the RNA-primed RNA nucleotidyltransferase activity of infected and uninfected Krebs II cells, cell nuclei, and cytoplasm was measured over a period of 7 to 9 hours after infection. In the preparations from the uninfected cells, nuclei, and cytoplasm, the transferase activity remained constant or declined slowly to levels 25 to 50% below the initial values and the limits of activities of the control preparations are indicated by the solid lines (Fig. 4). During the same period, the activity of the enzyme preparations from infected cells, cell nuclei, and cytoplasm either remained constant or rose to values above the initial controls. Absolute increases in the RNA-primed incorporation of UMPS of up to 96% above the zero time controls were observed in infected cells within 1 to 5 hours of infection. The extent and duration of this increased activity in infected cells was dependent on whether the enzymes were obtained from whole cells or cell fractions. It is significant that nearly all the values of enzyme activity from infected cells, particularly at the later times, fall above the limits shown for the uninfected cells.

The increase in transferase activity in the infected cells was clearly related to viral growth since it was only observed in experiments where viral replication could be demonstrated, e.g. by increased hemagglutinin titer or cell death, and in experiments when, for one reason or another, the virus failed to grow, no increase in transferase activity could be shown.

In the absence of added primer RNA, no differences were observed in the transferase activities of infected and uninfected cells.

**DISCUSSION**

Several enzyme systems that catalyze the formation of polynucleotides have been described (17, 18). Polynucleotide phosphorylase catalyzes the formation of polynucleotides from the ribonucleoside 5'-diphosphates and only shows a requirement for primer when highly purified. DNA-dependent RNA nucleotidyltransferases that require all four ribonucleoside 5'-triphosphates and direct the synthesis of RNA chains with base compositions determined by the DNA primer have been extensively studied in bacterial systems (19) and similar enzymes have been demonstrated in mammalian tissues (17, 18, 20, 21). Recently there has been considerable interest in RNA-dependent mechanisms for the biosynthesis of RNA (15, 17, 18, 20-22) and an enzyme has been obtained from *Micrococcus lysodeikticus* (22) that catalyzes the formation, from ribonucleoside 5'-triphosphates, of polynucleotides with base compositions related to those of the synthetic polynucleotides used as primers. The physiological significance of these systems for RNA-dependent synthesis of RNA is not known but they may assume particular importance in cells infected with RNA viruses. Such viruses, by analogy with tobacco mosaic virus (23-25), presumably carry their genetic information in RNA and indeed infectious RNA has been isolated from several mammalian viruses (26). It might be expected therefore that the viral RNA itself should serve as the template for the replication of RNA and protein and it is precisely in such a situation that an RNA-primed RNA nucleotidyltransferase would be required.

There is already evidence to suggest that the replication of viral RNA within certain mammalian systems is independent of either new DNA synthesis or of DNA-dependent synthesis of RNA. Thus viral replication has been shown to continue in the presence of inhibitors of DNA synthesis such as halogen substituted nucleotides (4, 5) and actinomycin D (6) at concentrations sufficient to inhibit both DNA nucleotidyltransferase and DNA-primed RNA nucleotidyltransferase. In a recent note (27), Baltimore and Franklin have demonstrated striking increases in the activity of an RNA nucleotidyltransferase from the microsomes and mitochondria of L cells infected with Mengo virus. This enzyme shows a pH optimum in the region of 8 to 9, a requirement for Mg2+ ions, and an inhibition by Mn2+ ions. Moreover it is independent of added DNA and is relatively insensitive to actinomycin D. It is therefore similar in many respects to the RNA-primed RNA nucleotidyltransferase that we have described which is also resistant to actinomycin D.

The present experiments provide evidence for the independent existence of an RNA-primed RNA nucleotidyltransferase in
Krebs II ascites tumor cells. The observation that the activity of this enzyme increases on infection of the cells with encephalomyocarditis virus is further evidence in support of the view that the synthesis of viral RNA may take place on an RNA template under the influence of an RNA-primed RNA nucleotidyltransferase.

The activities of deoxyribonucleic acid (DNA) nucleotidyltransferase, DNA-dependent ribonucleic acid (RNA) nucleotidyltransferase, and RNA-dependent RNA nucleotidyltransferase in preparations from uninfected Krebs II mouse ascites tumor cells and cells infected with encephalomyocarditis virus have been measured. Evidence is presented for the existence in both infected and uninfected cells of two distinct RNA nucleotidyltransferases, one dependent on the presence of DNA and the other on RNA.

No differences were detected in the activities of DNA nucleotidyltransferase and DNA-primed RNA nucleotidyltransferase of infected and uninfected cells. However, the activity of the RNA-dependent RNA nucleotidyltransferase from the infected cells was found to increase significantly within a few hours of infection.

Acknowledgments—We should like to express our thanks to Professor J. N. Davidson, F.R.S., for his interest in this work and for providing the facilities with the aid of grants from the Jane Coffin Childs Memorial Fund for Medical Research, the British Empire Cancer Campaign, and the Rankin Fund of the University of Glasgow. Thanks are also due to Miss S. Bowcott for skilled technical assistance. One of us (R. M. S. S.) acknowledges receipt of a grant from the Royal Society for the purchase of equipment.

References

The Biosynthesis of Ribonucleic Acid following Infection with a Ribonucleic Acid Virus
M. J. Cline, R. Eason and R. M. S. Smellie


Access the most updated version of this article at http://www.jbc.org/content/238/5/1788.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/238/5/1788.citation.full.html#ref-list-1