Ribonucleic Acids in Virus-infected and Uninfected Krebs II Ascites Tumor Cells

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Two lines of evidence support the view that the replication of ribonucleic acid containing animal viruses proceeds via RNA-directed synthesis of RNA. It has been shown that RNA viruses continue to replicate under conditions in which synthesis of deoxyribonucleic acid and the DNA-dependent synthesis of RNA are artificially suppressed (1-3). Furthermore, in certain mammalian cell lines there is evidence for enhanced RNA-dependent synthesis of RNA after infection with an RNA virus (4, 5).

The present investigations were undertaken in order to determine whether during the replication of an RNA-containing animal virus there is evidence for the early formation of a rapidly labeled unstable RNA analogous to that formed in bacterial cells after infection with a DNA virus. A rapidly labeled RNA distinguishable by its sedimentation characteristics from ribosomal and transfer RNA has been identified both in uninfected Krebs II mouse ascites tumor cells and in cells infected with the RNA-containing virus of mouse encephalomyocarditis. However, the sedimentation characteristics of this RNA from infected cells were indistinguishable from those of RNA in uninfected cells, and its synthesis could be suppressed by actinomycin D without interfering with the replication of the virus. This would suggest that the virus RNA can serve as the template for the synthesis of proteins involved in the initial stages of viral replication.

EXPERIMENTAL PROCEDURE

Methods—The methods for the propagation, growth, and assay of encephalomyocarditis virus in Krebs II mouse ascites tumor cells were essentially those described by Martin et al. (6).

DNA was prepared from calf thymus gland and from Krebs II and Landshutz mouse ascites tumor cells by the method of Kay, Simmons, and Dounce (7).

RNA was prepared from Krebs II ascites tumor cells as follows: 1 to 2 ml of washed packed cells (approximately 4 x 10^8 cells) were suspended in 0.8 ml of 2.5% bentonite in 0.01 M sodium acetate buffer, pH 5.2, and the aqueous phase was removed and the RNA was extracted from them and subjected to analysis. The combined aqueous extracts were shaken for 10 minutes with an equal volume of water-saturated phenol and centrifuged at 10,000 x g for 10 minutes, and the aqueous phase was removed. This was made 2% with respect to sodium acetate and the RNA was precipitated by the addition of 2 volumes of ice-cold ethanol. The RNA was collected by centrifugation in the cold for 30 minutes at 000 x g and dissolved in 10 ml of Buffer A (0.01 M Tris buffer, pH 7.5, 0.001 M with respect to MgCl_2). Pancreatic DNase (Sigma, crystalline, 10 to 20 μg) was added to the solution, which was then incubated for 15 minutes at 37°C. The solution was cooled; the RNA was precipitated by the addition of 2 volumes of ice-cold ethanol and after collection by centrifugation was dissolved in 5 to 10 ml of Buffer B (0.01 M sodium acetate, pH 5.2, 0.05 M NaCl, and 0.001 M MgCl_2) and dialyzed for 18 hours against two changes of 7 liters of this buffer. The dialyzed solution was clarified by centrifugation and the RNA was precipitated by the addition of 2 volumes of ice-cold ethanol and collected by centrifugation at 600 x g for 30 minutes. The final RNA preparation was dissolved in 1 to 2 ml of Buffer B and washed three times with ether, and the ether was blown off with nitrogen.

Incubation Conditions—In experiments on the incorporation of [H]-uridine and [P]-orthophosphate into the RNAs of uninfected and encephalomyocarditis virus-infected Krebs II ascites tumor cells, two samples of the cells were suspended at a concentration of 10^6 cells per ml in Earle's solution. One sample was infected with encephalomyocarditis virus (6) and both were allowed to stand at room temperature for 20 minutes before being transferred to a shaking incubator at 37°C. Either 30 or 60 minutes after infection, [H]-uridine or [P]-orthophosphate was added to both samples at final concentrations of 5 μc per ml and 20 μc per ml, respectively. At various time intervals thereafter cells were removed and the RNA was extracted from them and subjected to analysis.

In experiments with actinomycin D, three identical suspensions of Krebs II ascites tumor cells were prepared in Earle's solution at a concentration of 5 x 10^6 cells per ml. Actinomycin D was added to two of these at a final concentration of 10 μg per ml at room temperature 45 minutes before infection, and immediately before infection all three samples were diluted 5 times with Earle's solution. Encephalomyocarditis virus was added to one suspension 6 and all three samples were maintained at room temperature for 20 minutes before being transferred to a shaking incubator at 37°C. Sixty minutes after infection, [H]-uridine (7 μc per ml) was added and aliquots of the cells were removed at 80, 120, 240, 270, 330, and 420 minutes for isolation and analysis of the RNA.
Isotope Determinations—Tritium-labeled samples were assayed in a Packard Tri-Carb liquid scintillation spectrometer with a scintillator consisting of Scintimix NE 572 (Nuclear Enterprises, Edinburgh) dissolved in scintillation grade dioxane. Under the conditions employed it was not necessary to correct for quenching.

3P-Labeled samples in solution were usually dried on stainless steel planchets and counted in a Nuclear-Chicago gas flow counter fitted with a thin end window. No correction for self-absorption was necessary. In some experiments the 3P-labeled samples were assayed in liquid scintillator under conditions similar to those employed for tritium.

Phosphorolysis Experiments—Polyribonucleotide phosphorolysis was prepared from spray-dried Micrococcus lysodeikticus cells (California Corporation for Biochemical Research) by the method of Steiner and Beers (8). This enzyme was employed in the phosphorolysis of 32P- and 3H-labeled RNA from infected cells and encephalomyocarditis virus (8).

Ultracentrifugal Studies—Sedimentation analyses of RNA preparations were performed by centrifugation in linear sucrose density gradients (20 to 5% sucrose in Buffer B) in the SW-20 rotor of a Spinco model L ultracentrifuge (9). Centrifugation was usually carried out for 12 hours at 23,000 r.p.m. at 0°C.

Sedimentation analyses of RNA and DNA in CsCl density gradients were carried out by the method of MesseIon, Stahl, and Vinograd (10) at a CsCl density of 1.73 g per ml. Centrifugation was performed in the SW-20 rotor at 35,000 r.p.m. at 24°C for 48 hours. CsCl (Analar) was obtained from Hopkins and Williams, Ltd., and was first freed from material absorbing at 260 nm by passage in dilute solution through a charcoal column. The density of the CsCl solution was determined with an Abbe refractometer.

Annealing of RNA and DNA—The procedure for annealing RNA and DNA mixtures was that described by Hall and Spiegelman (11). The 40-S peak of RNA from uninfected Krebs II ascites tumor cells was isolated from sucrose density gradients, dialyzed against 0.015 M NaCl-0.001 M sodium citrate, pH 7.8, to remove sucrose, and concentrated 10 times by lyophilization. The resulting solution was heated to 100°C for 5 minutes, rapidly cooled to denature the RNA, and mixed with DNA (in 0.15 M NaCl-0.01 M sodium citrate, pH 7.8) from Krebs II or Landschutz ascites tumor cells or calf thymus at a final concentration of 100 μg per ml. The mixture was heated to 57°C for 2 hours, allowed to cool slowly to 20-30°C (11), and analyzed in CsCl density gradients.

Materials—Actinomycin D was a gift of Merck, Sharp and Dohme, Inc.; 3H-uridine with a specific activity of 1.57 curies per mmole and carrier-free 32P-orthophosphate were purchased from the Radiochemical Centre, Amersham, England.

RESULTS

Density Gradient Analysis of Labeled RNA from Infected and Uninfected Cells—Krebs II ascites tumor cells were suspended in Earle's solution and incubated with 3H-uridine for varying periods of time. RNA was extracted from the cells as already described and centrifuged in sucrose density gradients. Three peaks of optical density at 256 nm were identified, corresponding to 30-S, 19-S, and 4-S RNAs. After a 20-minute exposure to 3H-uridine, radioactivity was detected in two peaks (Fig. 1), one sedimenting in a position corresponding to approximately 40-S, and the other, to 4-S RNA. After incubation of the cells for 90

and 270 minutes with 3H-uridine, radioactivity was distributed throughout the three peaks of ultraviolet-absorbing material in addition to the rapidly sedimenting peaks (Fig. 2). Similar patterns of labeling of RNA have been observed by Scherrer and Darrell (12) after the incubation of IleLa cells with 3C-uridine, and we have found qualitatively similar patterns in the present study when 3P-orthophosphate was used to label the RNA except that after the shortest exposures (20 minutes) little or no radioactivity was detected in the 40-S, 30-S, or 19-S regions.

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Hybridization of RNA and DNA—When the 40-S peak of RNA from Krebs II ascites tumor cells labeled by a 20-minute exposure to 3H-uridine was annealed with calf thymus or Landschutz mouse ascites tumor cell DNA, no radioactivity was detected in the region of the CsCl gradient where the DNA sedimented (Fig. 3). However, when the same RNA was annealed with Krebs II mouse ascites tumor cell DNA, significant amounts of radioactivity, corresponding to between 30 and 40% of the total radioactivity of the RNA, sedimented in the region of the DNA (Fig. 3). This RNA therefore exhibits two essential properties...
of "messenger" RNA in being rapidly labeled and in forming hybrids only with the homologous DNA.

Effect of Actinomycin D on Labeling of RNA and on Virus Replication—The influence of actinomycin D on the incorporation of H-uridine into the RNAs of infected and uninfected Krebs II ascites tumor cells is illustrated in Figs. 4 and 5. RNA obtained from cells at 80, 120, 240, 270, 330, and 420 minutes after infection was analyzed but only the results for the 80-minute and 330-minute times are presented, since these are representative of the two types of result obtained.

In uninfected cells treated with actinomycin D, no significant radioactivity was detected in the 30-S and 19-S RNA peaks at any of the time intervals, although a small amount of activity was observed in the 4-S region at each time (Figs. 4 and 5). The total amount of radioactivity incorporated into the isolated RNA of these cells after 4 to 6 hours of incubation with H-uridine was less than 5% of that incorporated into controls not treated with actinomycin D.

In the cells infected with encephalomyocarditis virus and treated with actinomycin D, all the H-uridine or P-orthophosphate incorporated into the isolated RNA during the period up to 4 hours after infection was also located in the 4-S region of the sucrose density gradients (Fig. 4), and the extent of labeling of the RNA was quantitatively similar to that in the uninfected, actinomycin D-treated cells during this time. At 4½ hours post infection, however, a new peak of radioactivity appeared in the RNA from the infected cells. This peak migrated in advance of the 30-S peak at approximately 35 S (Fig. 5), a position that corresponds to that described for infectious RNA (13) and mature virus RNA. The radioactivity in this peak increased steadily with time of incubation up to at least 8 hours after infection.

Analysis of 4-S RNA Labeled in Presence of Actinomycin D—The radioactivity in the 4-S region of the sucrose density gradients of RNA from cells labeled with H-uridine in the presence of actinomycin D was insoluble in 5% trichloroacetic acid and became acid-soluble after treatment with dilute alkali. It seemed probable that this peak represented the transfer RNA of the cells that had become labeled by a non-DNA-dependent addition of cytidine residues formed from H-uridine. To test this possibility, the 4-S peak of RNA was isolated, concentrated by lyophilization, and analyzed for the distribution of radioactivity among the ribonucleoside 3'(2')-monophosphate residues. This varied to some extent depending on the duration of the...
exposure to $^3$H-uridine, but in every case the major proportion of the activity was found in cytidine 3'$(2'2')$-monophosphate (Table 1).

Analysis of $^{35}$S RNA Component Synthesized after Encephalomyocarditis Virus Infection—If the replication of viral RNA occurs on an RNA template, it would be expected that, at some stage after infection, a strand of RNA would be formed complementary in base sequence to the invading viral RNA. This might exist as an independent strand of RNA or might be associated with the viral RNA in the form of a double stranded molecule.

Virus was grown in Krebs II ascites tumor cells in the presence of actinomycin D and either $^3$H-uridine or $^{32}$P-orthophosphate. There was a progressive increase in the amount of radioactivity incorporated into the $^{35}$S RNA band during the period 4$^{1/2}$ to 8 hours after infection, and $^{35}$S RNA peaks were isolated from sucrose gradients 6 and 8 hours after infection. Labeled RNA from the mature virus was prepared from the medium in which the cells were suspended 21 hours after infection. When the three labeled RNA preparations were subjected again to sucrose density gradient analysis under identical conditions, the $^{35}$S RNA from the cells 6 and 8 hours after infection and the mature virus RNA behaved in precisely the same manner, suggesting similar molecular configurations.

In an attempt to distinguish between the hypothetical single stranded RNA of the mature virus and the hypothetical double stranded RNA of the immature virus, polynucleotide phosphorlyase was employed. It is known that polynucleotide phosphorlyase readily attacks single stranded synthetic polyribonucleotides but that multistranded polyribonucleotides are much more resistant (15). The phosphorolysis of complex polyribonucleotides such as RNA by this enzyme is less well understood.

Fig. 4. Sedimentation analysis in sucrose density gradients of RNA from uninfected; uninfected, actinomycin D-treated; and encephalomyocarditis virus-infected, actinomycin D-treated Krebs II mouse ascites tumor cells labeled with $^3$H-uridine during the interval 60 to 80 minutes after infection.

Fig. 5. Sedimentation analysis in sucrose density gradients of RNA from uninfected; uninfected, actinomycin D-treated; and encephalomyocarditis virus-infected, actinomycin D-treated Krebs II mouse ascites tumor cells labeled with $^3$H-uridine during the interval 60 to 330 minutes after infection.
TABLE I

Distribution of radioactivity in ribonucleoside 3'(2')-monophosphates obtained on alkaline hydrolysis of 35S RNA from Krebs II cells incubated with 3H-uridine in presence of actinomycin D

The conditions of incubation were as described in Fig. 4. The 4-S peak of radioactivity was isolated from the sucrose gradient, dialyzed, concentrated by lyophilization, and digested with 0.3 n KOH for 18 hours at 37° to yield the ribonucleoside 3'(2')-monophosphates. These were separated by electrophoresis on filter paper (14), eluted, and counted in a liquid scintillation spectrometer.

<table>
<thead>
<tr>
<th>Ribonucleotide</th>
<th>20-Minute exposure</th>
<th>270-Minute exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytidine 3'(2')-phosphate</td>
<td>65</td>
<td>91</td>
</tr>
<tr>
<td>Uridine 3'(2')-phosphate</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>Adenosine 3'(2')-phosphate</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Guanosine 3'(2')-phosphate</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
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but nevertheless it would appear that species of RNA with a high degree of secondary structure, such as transfer RNA, are resistant to phosphorolysis (16).

Preliminary experiments with polynucleotide phosphorylase showed that the enzyme phosphorolysed complexes of polyadenylic acid and polyuridylic acid to only 30 to 50% of the extent to which it attacked the individual homopolymers. As can be seen from Fig. 6, however, the 35-S RNA peaks from the infected cells 6 and 8 hours after infection and the RNA from the mature encephalomyocarditis virus were all rapidly and completely phosphorolysed. Denaturation of the RNA preparations by heating to 100° for 10 minutes followed by rapid cooling had no effect on the extent or rate of the reaction.

DISCUSSION

The biochemical events occurring in cells infected with RNA-containing viruses are of considerable interest since in such systems the normal mechanisms of information transfer from DNA to messenger RNA and then to protein are bypassed to some extent.

The available evidence from bacterial cells infected with DNA-containing viruses, such as Escherichia coli infected with bacteriophage T2, indicates that the viral DNA is used as a template for the synthesis of a virus-specific messenger RNA by the DNA-dependent RNA nucleotidyltransferase of the host cell and that this messenger RNA in turn acts as a template for the synthesis of specific enzymes and proteins concerned with the synthesis of new viral DNA and protein (17-19).

In animal systems infected with RNA-containing viruses (20, 21), there is evidence of a period shortly after infection when subsequent synthesis of RNA is dependent on the synthesis of protein. Furthermore, an increase in the synthesis of protein has been demonstrated in Krebs II ascites tumor cells shortly after infection with encephalomyocarditis virus (6), and it has been demonstrated that certain mature virus RNAs can behave as messengers in systems synthesizing proteins in vitro (22-24). These observations are consistent with our finding (4, 5) that there is an increase in the activity of RNA-dependent RNA nucleotidyltransferase in Krebs II cells 1 to 2 hours after infection with encephalomyocarditis virus.

A variety of schemes may be postulated for the mechanism of synthesis of virus RNA in cells infected with an RNA virus, and several of these are illustrated in Fig. 7. The mechanisms can be broadly divided into two categories, depending on whether the initial phase involves the utilization of the host protein-synthesizing system and the infecting viral RNA to form a new RNA-dependent RNA nucleotidyltransferase or whether the host RNA-dependent RNA nucleotidyltransferase is employed to make a complementary RNA or a direct copy RNA from the infecting viral RNA.

Route 1 indicates synthesis of new virus RNA by a direct copy

![Fig. 6. Phosphorolysis by polynucleotide phosphorylase of 3P labeled RNA isolated from mature encephalomyocarditis virus and from actinomycin D-treated Krebs II ascites tumor cells 6 and 8 hours after infection with encephalomyocarditis virus. 3P-Labeled RNA was incubated with 310 μmoles of KCl, 3.1 μmoles of MgCl₂, 31 μmoles of K₂HPO₄, 154 μmoles of Tris buffer, pH 8.5, and 0.2 ml of polynucleotide phosphorylase in a total volume of 2.2 ml at 37°. Aliquots (1 ml) were removed, mixed with 0.1 ml of carrier yeast RNA (1 mg per ml), and precipitated with 5% HClO₄. The supernatant fluid was diluted and samples were taken for 3P assay.]
Figure 7. Possible mechanisms of replication of virus RNA

Mechanism from the infecting viral RNA, utilizing the virus-induced RNA-dependent RNA nucleotidyltransferase. The formation of viral RNA from a complementary RNA by a conservative mechanism in which the complementary RNA behaves catalytically or by a semiconservative mechanism in which an equal number of molecules of complementary RNA and new virus RNA are produced is indicated by Route 2.

Route 3 illustrates the possibility of formation of new virus RNA by a direct copy mechanism utilizing the host cell RNA-dependent RNA nucleotidyltransferase, and Routes 4 and 5 would be dependent on the formation of an intermediary complementary RNA from the infecting viral RNA by the host cell RNA-dependent RNA nucleotidyltransferase. New RNA-dependent RNA nucleotidyltransferase could be formed by use of this complementary RNA as a template, and virus RNA could be synthesized by a direct copy mechanism from the infecting virus RNA. Although it seems most unlikely, the point should be made that the complementary RNAs in these pathways might themselves be infective and might constitute the new viral RNA.

The formation of new virus RNA by mechanisms such as Routes 4 and 5 (Fig. 7) involving the utilization of a host cell RNA-dependent RNA nucleotidyltransferase followed by a virus-induced RNA nucleotidyltransferase seems unlikely on general grounds. If, however, such a pathway were operative, a rapidly labeled RNA, analogous to the messenger RNA of E. coli cells infected with bacteriophage T2, would be expected to occur early after infection of the Krebs II cells with encephalomyocarditis virus and before the appearance in the cells of increased levels of RNA-dependent RNA nucleotidyltransferase activity. No such RNA would be required at an early time interval after infection if the mechanism of biosynthesis of new viral RNA proceeded by Routes 1 or 2.

Normal Krebs II ascites tumor cells contain a rapidly labeled, high molecular weight RNA exhibiting a significant degree of complementarity with homologous but not heterologous DNA (Fig. 3). This is a particularly significant observation, since it indicates the existence in mammalian cells of a species of RNA that is not only rapidly labeled but which contains regions of base sequence complementary to those in the cell DNA, thus exhibiting one of the most important characteristics of a messenger RNA. An RNA peak with similar, indeed indistinguishable, sedimentation characteristics is also found in Krebs II cells infected with encephalomyocarditis virus. In both infected and uninfected cells, this rapidly labeled peak of RNA is eliminated by pretreatment of the cells with actinomycin D (Fig. 4). In the presence of actinomycin D, the synthesis of RNA in both infected and uninfected cells is abolished during the first 4 hours after infection except for some terminal labeling of transfer RNA. Nevertheless the encephalomyocarditis virus continues to replicate, and synthesis of RNA can be demonstrated commencing about 4½ hours after infection (Fig. 5). There is therefore no evidence from our experiments for the early synthesis of RNA utilizing existing host cell RNA-dependent RNA nucleotidyltransferase, and on the basis of these results we conclude tentatively that Pathways 4 and 5 are not involved in the synthesis of the new viral RNA. Moreover, since synthesis of protein has been shown to be an essential prerequisite for the formation of new virus RNA (21), Route 3 can be discarded. It appears, therefore, that synthesis of new virus RNA must follow Routes 1 or 2, and this is supported by our observation (4, 5) of increased levels of RNA-dependent RNA nucleotidyltransferase within 1 to 2 hours of infection of the cells with encephalomyocarditis virus and from the finding (24) that at least some animal virus RNAs are capable of acting directly as messengers in the synthesis of virus-specific proteins.

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The diagram represents the possible mechanisms of replication of virus RNA. The process begins with the infecting virus RNA, which is then utilized by a host protein-synthesizing system. The RNA-dependent RNA nucleotidyltransferase is induced by the viral RNA, leading to the formation of complementary RNA. This complementary RNA then serves as a template for the synthesis of new virus RNA, which can either be achieved by direct copy, conservative, or semiconservative replication. The diagram illustrates these pathways, each labeled with numbers corresponding to the text explanation.
SUMMARY

Sucrose density gradient analyses have been carried out on the ribonucleic acids isolated from uninfected Krebs II ascites tumor cells and from cells infected with the ribonucleic acid virus of mouse encephalomyocarditis after the incubation of the cells for varying times with 3H-uridine. Three peaks of optical density at 256 mμ, corresponding to 30 S, 10 S, and 4 S ribonucleic acids, were demonstrated in both uninfected and infected cells. In addition a rapidly labeled peak of radioactivity corresponding in position to approximately 40 S ribonucleic acid was demonstrated in both infected and uninfected cells. No differences were observed in the nature of this material in the infected and uninfected cells.

No hybrid formation was observed when this rapidly labeled 40 S peak from uninfected cells was annealed with preparations of deoxyribonucleic acid from calf thymus or Landschutz mouse ascites tumor, but a substantial degree of hybridization occurred when the ribonucleic acid was annealed with homologous deoxyribonucleic acid.

Actinomycin D treatment of both uninfected and infected Krebs II cells abolished the incorporation of 3H-uridine into the 40 S, 30 S, and 19 S peaks of ribonucleic acid and greatly decreased incorporation into the 4-S peak at time intervals up to 4 hours after viral infection. Thereafter, however, a new peak of radioactivity sedimenting at approximately 35 S appeared in the ribonucleic acids from the infected cells. The actinomycin D-resistant labeling of the 4 S peak of ribonucleic acid was found to arise from the incorporation of cytidine residues derived from the 3H-uridine. This presumably represents non-deoxyribonucleic acid-dependent terminal and sub-terminal additions to transfer ribonucleic acid.

Analysis of the actinomycin D-resistant 35-S peaks of ribonucleic acid from infected cells provided no evidence for the existence of a double stranded molecule.

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