Synthesis of Ribonucleic Acid in Cells Infected with LSc Poliovirus at Elevated Temperatures¹

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The synthesis of viral ribonucleic acid (RNA) was detected within 2 hr after infection with LSc poliovirus at 35 C. This RNA eluted as a single peak with 0.9 M NaCl on methylated albumin celite columns, was sensitive to ribonuclease, precipitated in the presence of 2 M LiCl, and had an S_{20} value at 34 ± 2 in linear sucrose gradients. When cells were infected at 39 to 40 C, there was also early synthesis of RNA. However, 2 hr after infection this synthesis was drastically inhibited. The absence of net RNA synthesis at 39 to 40 C during the late stages of infection was not caused by rapid degradation of newly formed RNA, since the RNA produced between 1 and 2 hr at 39 to 40 C was still present 3.5 hr after infection. There was a 3 $\log_{10}$ inhibition in the production of infectious virus when *p*-fluorophenylalanine was present in the medium at a concentration of 25 μ g/ml. This concentration of analogue had little effect upon the production of viral polymerase and viral RNA. Virus grown in the presence of analogue at a concentration of 10 μ g/ml exhibited increased heat sensitivity compared to control virus. However, viral polymerase exhibited no change in sensitivity to heat or manganese when cells were exposed to 25 μ g of *p*-fluorophenylalanine per ml during infection. *p*-Fluorophenylalanine had a relatively selective effect on viral capsid protein but did not reverse the inhibition of synthesis of viral RNA at 39 to 40 C.

The LSc strain of type 1 poliovirus cannot replicate at 39 to 40 C. The defect in the developmental cycle of the virus occurs after the first 2 hr of infection, because shifting infected cells to 35 C after 2 hr of incubation at 39 to 40 C results in a near normal yield of virus with no delay in virus synthesis (17). In addition at the elevated temperatures there is a 95% inhibition in the synthesis of both poliovirus polymerase and viral ribonucleic acid (RNA) during the late stages of infection (17). The reason for the inhibition of synthesis of viral-induced macromolecules has not been determined. First, it is possible that the production of viral RNA occurs to some extent, but that activation of nucleases at higher temperatures leads to the rapid degradation of newly formed RNA. Second, blockage of synthesis of viral RNA could reflect an abnormal functioning of control mechanisms at elevated temperatures. Good evidence is available that indicates strict regulation of protein synthesis among RNA phages and poliovirus (7, 13, 15). Lodish and Zinder suggested that the coat protein of RNA phages binds to viral RNA during the course of

infection and represses the translation of certain viral cistrons (13). It is conceivable that the capsid protein of LSc poliovirus operates as a malfunctioning repressor at 39 to 40 C. These hypotheses have been examined in the following report.

MATERIALS AND METHODS

Cell cultures and virus. HeLa cells were grown as monolayer cultures in 32-oz (0.946 liter) bottles in Hanks' medium. The medium and methods for handling cells have been described (21). The LSc strain of type 1 poliovirus was used.

Infection of cells and extraction of RNA. The procedures for infecting cells in the presence of tritiated uridine and for extracting RNA have been published (17). Usually, 10^6 cells were used per sample; these cells were infected at a ratio of 200 plaque-forming units (PFU) of virus per cell in the presence of 150 to 400 μ Ci of uridine-5-³H (19.5 c/mmole; Schwartz Bio Research Inc., Orangeburg, N.Y.).

Methylated-albumin chromatography. Methylated albumin (Armour, fraction V) was prepared by the method of Mandell and Hershey (16), and methylated albumin celite (MAK) columns were prepared by the procedure of Sueoka and Cheng (19). The chromatographic procedure and the method for measuring radioactivity were previously outlined (17).

Sucrose gradient centrifugation. Linear sucrose gradients (30 to 10%) were prepared by mixing 2.2

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ml of 30% sucrose and 2.2 ml of 10% sucrose in a Buchler apparatus. Sucrose was dissolved in 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.7, which also contained 0.1 M NaCl, 1 mM ethylenediaminetetraacetate, and 0.1% sodium dodecylsulfate (SDS). Samples containing 100 to 200 μ g of RNA in 0.25 ml of 10 mM Tris buffer were centrifuged for 4 hr at $122,000 \times g$ at 22 to 25 C in an SW65 rotor of the model L2 Beckman ultracentrifuge. Thirty 0.15-ml fractions were collected after puncturing the bottom of each tube. RNA was precipitated from each sample with 4 ml of 5% trichloroacetic acid, collected on filters, and counted. Recovery of radioactivity was at least 80%. Ribosomal RNA of *Escherichia coli* (23S; Miles Laboratories, Inc., Elkhart, Ind.) was included in replicate samples as a standard.

Preparation and assay of poliovirus polymerase. Crude enzyme extracts were prepared by the water homogenization procedure of Baltimore and Franklin (3). Details of the assay procedure have been previously published (21).

Heat inactivation of virus. Samples (0.3 ml) of virus in medium were added to 100-mm tubes and were placed in a water bath at 50 ± 1 C. At intervals, samples were removed from the water bath, diluted 10-fold with cold medium, and titrated by the plaque procedure. The medium employed in these experiments was balanced salt solution (BSS) that contained 7% dialyzed calf serum.

RESULTS

Production of RNA in virus-infected cells. To establish the pattern of RNA synthesis in poliovirus-infected cells, six flasks, containing 10^8 cells each in 25 ml of medium, were infected at a ratio of 200 PFU/cell. Three flasks were held at 35 C, and the remaining three were held at 39 to 40 C. In the cultures incubated at 39 to 40 C, virus attachment was also permitted to occur at 39 to 40 C. Between 1 and 2, 2 and 3, and 3 and 4 hr after infection, cultures were pulse-labeled with 150 μ Ci of 3 H-uridine (19.5 c/mmole). Actinomycin D at a concentration of 5 μ g/ml was present during the first hour of infection. This concentration of drug did not inhibit virus development, but eliminated cellular RNA synthesis so that the synthesis of viral RNA could be detected. A 1-hr period of exposure of actinomycin D was a standard procedure, since additional incubation with this compound did not cause any further significant blockage of cellular RNA synthesis.

After extraction of RNA, samples were passed through freshly prepared MAK columns. Figures 1 to 3 show the results of one study which was reproduced several times.

Actinomycin D completely eliminated cellular RNA synthesis in uninfected cells (Fig. 1). Between 1 and 2 hr after infection at 35 C, RNA that eluted from the columns with 0.9 M NaCl was produced (Fig. 1). Because elution volumes of 4.0

FIG. 1. Incorporation of 3 H-uridine by poliovirus-infected cells 1 to 2 hr after infection. Incubation at 35 C, 306 μ g of RNA, 520 counts/min (●); incubation at 39 to 40 C, 313 μ g of RNA, 344 counts/min (×); uninfected cells plus actinomycin D, 350 μ g of RNA, 31 counts/min (Δ). Each fraction is plotted as a point. The 0.9 M RNA begins to elute in the last 0.8 M fraction because 4-ml elution volumes were used on columns with bed volumes of 3.5 ml.

ml were used in columns with 3.5-ml bed volumes, the last 0.8 M NaCl eluate contained some of the RNA that elutes with 0.9 M NaCl. The amount of RNA that eluted with 0.9 M NaCl increased significantly between 2 and 3 hr (Fig. 2). By 3 to 4 hr, the radioactivity in the virus-induced RNA had increased 180-fold over that found during the 1 to 2 hr labeling period (specific activity of RNA, 1.7 counts per min per μ g versus 310 counts per min per μ g). Specific activities of RNA were determined on the total extract of RNA in which unlabeled cellular RNA was the major component.

Viral-induced RNA was also synthesized 1 to 2 hr after infection at 39 to 40 C (Fig. 1). The quantity of RNA that eluted from the columns with 0.9 M NaCl was consistently 30 to 50% lower than the quantity found at 35 C. Between 2 and 3 and 3 and 4 hr after infection, there was no significant synthesis of RNA at 39 to 40 C (Fig. 2 and 3). The synthesis of RNA during early stages of infection at 39 to 40 C is in agreement with previous growth-curve experiments (17). In these studies, incubation of cultures at 39 to 40 C during the first 2 hr of infection had very little effect on the virus growth curve (see Fig. 3; reference 17).

Nature of virus-induced RNA. Numerous studies have been performed with MAK columns and poliovirus-infected cells (5, 6, 11, 14, 18). These experiments indicated that infectious,

single-stranded RNA elutes anywhere between 0.77 and 1.2 M NaCl. The temperature of the column and the preparation of serum albumin can lead to differences in elution profile (11).

To determine whether the RNA that eluted with 0.9 M NaCl was single-stranded or double-stranded, samples of RNA were treated with 7.5 μ g of ribonuclease (Sigma Chemical Co., St. Louis, Mo., 5 $\times$ crystallized) per ml for 15 min at 35 C (0.5 M NaCl, 0.05 M phosphate buffer, pH 6.8). After treatment with enzyme, undigested RNA was precipitated from the preparations with ethyl alcohol in the presence of 200 μ g of carrier RNA from uninfected cells. The RNA precipitates were collected by centrifugation, dissolved in 0.05 M phosphate buffer, and chromatographed. The viral-induced RNA was completely digested (Fig. 2 and 3). This method was sensitive enough to establish that over 95% of the RNA was hydrolyzed in samples containing less than 1,000 counts/min and over 99% was hydrolyzed in samples containing more than 10,000 counts/min.

To confirm the ribonuclease studies, RNA from the 0.9 M NaCl fractions was collected from the columns and 2-ml amounts of the RNA preparations were mixed with equal volumes of 4 M LiCl and incubated for 20 hr at 4 C. After incubation in the cold, samples were centrifuged for 15 min at 15,000 $\times$ g and radioactivity measurements were made on the supernatant fractions and sediments. Consistently, over 90%

FIG. 2. Incorporation of ^3H -uridine by poliovirus-infected cells 2 to 3 hr after infection. Incubation at 35 C, 145 μ g of RNA, 10,261 counts/min ($\bullet$); incubation at 39 to 40 C, 280 μ g of RNA, background counts ($\times$); the 35 C sample treated with 7.5 μ g of ribonuclease per ml of 0.5 M NaCl buffer (pH 6.8) for 15 min at 35 C ($\circ$).

FIG. 3. Incorporation of ^3H -uridine by poliovirus-infected cells 3 to 4 hr after infection. Incubation at 35 C, 145 μ g of RNA, 44,865 counts/min ($\bullet$); incubation at 39 to 40 C, 252 μ g of RNA, background counts ($\times$); the 35 C sample treated with 7.5 μ g of ribonuclease per ml of 0.5 M NaCl buffer (pH 6.8) for 15 min at 35 C ($\circ$).

of the counts were recovered in the sediments. Since 2 M LiCl is known to precipitate single-stranded RNA, this also indicates that the newly synthesized RNA was single-stranded (2).

Finally, the RNA that eluted with 0.9 M NaCl was centrifuged in linear sucrose density gradients. Ribosomal RNA (23S; Miles Laboratory) from *E. coli* was also centrifuged as a marker for determining the sedimentation value of the viral-induced RNA.

The viral-induced RNA sedimented as a single band with an S_{20} value of 34 ± 2 (Fig. 4). These data further support the conclusion that the viral RNA was single-stranded and had sedimentation properties similar to those found in mature poliovirus (1, 4).

Metabolic stability of early RNA. The data presented thus far indicate that, after 2 hr at 39 to 40 C, LSc poliovirus failed to induce a significant level of RNA synthesis. An alternative explanation is that after 2 hr at elevated temperatures RNA synthesis continued to some degree, but that localized activation or synthesis of nucleases caused rapid degradation of any new RNA. To test this possibility, the stability of early RNA was examined.

Cells were infected as before at 35 C and at 39 to 40 C and were pulse-labeled with 400 μ Ci of

FIG. 4. Sucrose gradient centrifugation of the 0.9 M peak RNA. A 108- μ g amount of RNA, 3,095 counts/min, was centrifuged at 122,000 $\times$ g for 4 hr in an SW65 rotor. The 23S arrow indicates the peak of *E. coli* 23S ribosomal RNA used as a standard.

3 H-uridine 1 to 2 hr after infection. After the pulse label, the cells were washed three times with 100-ml portions of cold BSS containing a 75-fold excess of unlabeled uridine. The cells were then resuspended in medium containing a 75-fold excess of unlabeled uridine, and each sample was divided into two portions. RNA was immediately extracted from one set of cultures, whereas the remaining set of cultures was incubated an additional 90 min before RNA was extracted and chromatographed.

Figure 5a shows the chromatographic profiles of RNA immediately after labeling and after an additional 90 min at 39 to 40 C. After further incubation in the absence of 3 H-uridine, there was a slight increase in labeled RNA (1.8 counts per min per μ g of RNA versus 2.5 counts per min per μ g). This presumably occurred because the washing procedure and the incubation of cells with unlabeled uridine were not sufficient to completely dilute out radioactive precursors in the acid-soluble pool of the cell. Figure 5a also shows the extent of RNA synthesis between 2 and 4 hr at 39 to 40 C when the cells were labeled with 200 μ Ci of 3 H-uridine. A small degree of RNA synthesis occurred. This synthesis is not noticeable in Fig. 2 and 3, because the peak of RNA contained only about 60 counts/min and could not be expressed significantly on those graphs. The study indicates that the early RNA, along with some new RNA, was present 3.5 hr after infection at the higher temperature. If nucleases were active, viral RNA in the 0.9 M NaCl eluate should have been reduced to a level lower than

the control labeled from 2 to 4 hr at 39 to 40 C. This was anticipated because, as will be shown below, the washing procedure reduced subsequent incorporation of radioactivity by a factor of 10. The 200 μ Ci used in the experiment between 2 and 4 hr represented much more radioactive uridine than was found in the cells after washing. Therefore, this labeling procedure should provide some information about the maximum level of RNA synthesis that can occur between 2 and 4 hr at 39 to 40 C.

At 35 C the results were substantially different from the results at 39 to 40 C. The stability of early RNA could not be measured because the washing procedure only eliminated 90% of the incorporation of label. The 90-min incubation period after the wash resulted in an increase in counts over early RNA (26 counts per min per μ g versus 2.7 counts per min per μ g of RNA). Without washing, the expected specific activity of the RNA would be at least 300 counts per min per μ g of RNA (Fig. 3). The data indicate that washing and the presence of cold uridine were not capable of completely eliminating radioactive uridine from the acid-soluble pool. It appears that

FIG. 5. Metabolic stability of RNA synthesized 1 to 2 hr after infection. (a) Incubation at 39 to 40 C. Pulse-labeled for 1 to 2 hr, 482 μ g of RNA, 843 counts/min ($\times$); 1.5 hr after removing label, 396 μ g of RNA, 969 counts/min ($\circ$); RNA labeled with 200 μ Ci of 3 H-uridine between 2 and 4 hr (Δ). (b) Incubation at 35 C. Pulse labeled for 1 to 2 hr, 696 μ g of RNA, 1,858 counts/min ($\bullet$); 1.5 hr after removing label, 550 μ g of RNA, 14,195 counts/min (Δ).

the metabolic stability of early RNA could be measured in Fig. 5a, because at 39 to 40 C there was severe inhibition of the synthesis of viral RNA.

One finding should be mentioned. A small fraction of RNA eluted with 0.8 M NaCl (Fig. 5). At first this was thought to represent a special type of RNA. However, unpublished studies now show that adding 500 μ g or more of RNA to the columns resulted in the elution of some of the RNA with 0.8 M NaCl. Aggregation of RNA has been shown to affect elution profiles on MAK columns (9), and this provides a possible explanation for the observation presented here.

Effect of *p*-fluorophenylalanine on the production of poliovirus, viral polymerase, and viral RNA. Several reports have shown that *p*-fluorophenylalanine (FPA) inhibits the formation of infectious poliovirus (8, 12). Concentrations of analogue that block poliovirus production 99% have relatively little effect upon the synthesis of viral RNA. The capsid protein of poliovirus and the virus particles are produced in the presence of inhibitor but are structurally altered, probably because of the incorporation of FPA (8).

Experiments were performed to determine the effect of FPA on the production of poliovirus, viral polymerase, and viral RNA. Cells were infected under the same conditions that were used to study RNA synthesis. Virus was allowed to adsorb to cells for 30 min. After the adsorption period, the cells were washed with BSS and resuspended in BSS containing 7% dialyzed calf serum. Various concentrations of a racemic mixture of FPA (Nutritional Biochemicals Corp., Cleveland, Ohio) were added to sets of cultures 30 min after infection, and the production of infectious virus and virus-induced macromolecules was determined by methods previously described (17).

The formation of infectious poliovirus was highly sensitive to inhibitor (Fig. 6). A concentration of FPA of 25 μ g/ml caused a 3 log₁₀ reduction in the yield of virus, whereas 50 μ g of analogue per ml completely eliminated the formation of new virus. The leveling of the curve was caused by virus that failed to undergo eclipse. On the other hand, the synthesis of viral polymerase and viral RNA was relatively resistant to FPA. Concentrations of 25 to 40 μ g/ml eliminated only 30 to 60% of the synthesis of these macromolecules.

Since it has been suggested that FPA is incorporated into poliovirus-induced proteins (8), experiments were performed to determine whether the characteristics of the virus and of viral polymerase were changed when virus was propagated in the presence of analogue. Virus was grown in

control cultures and cultures exposed to 10 μ g of FPA per ml. The conditions of infection were analogous to those already described. Cells were infected at a ratio of 200 PFU/cell, the medium contained 7% dialyzed serum, and inhibitor was added 30 min after infection. The heat sensitivities of control virus and virus obtained in the presence of analogue (FPA-virus) were determined (Fig. 7). After 1 min at 50 C, control virus lost about 90% infectivity, whereas FPA virus was far more sensitive. About 99.9% of the infectivity of FPA-virus was destroyed after a 1-min exposure to heat.

FIG. 6. Effects of various concentrations of FPA on the production of poliovirus (●), viral RNA (Δ), and viral polymerase (×). Semilog plot.

FIG. 7. Sensitivity of poliovirus (×) and FPA-virus (●) to heat. Samples of virus were exposed to 50 C for different lengths of time, diluted with cold medium, and titrated by the standard plaque procedure. Survival of infectivity is plotted on a semilog scale.

In addition, studies were performed to determine whether FPA altered the properties of poliovirus polymerase. Enzyme was extracted from infected cells that were incubated in the presence of 25 μg of FPA per ml. The sensitivity of enzyme to heat and manganese was determined (21). The heat sensitivity (Fig. 8A) and the sensitivity of enzyme to manganese (Fig. 8B) were similar to control enzyme. Other experiments were performed with 50 μg of FPA per ml, but the results were also negative.

Effect of FPA on RNA synthesis at 39 to 40 C. The previous experiments and the data of other investigators (8) show that FPA has a selective effect on viral capsid protein. If the capsid protein of virus functions as a repressor of the polymerase cistron, as previously suggested (13), and if the capsid protein behaves abnormally at 39 to 40 C, it might be possible to reverse the inhibition of RNA synthesis at 39 to 40 C with FPA. To test this, sets of cultures were infected with poliovirus at 35 C and at 39 to 40 C. Different concentrations of analogue were added to cultures 30 min after infection, and the synthesis of RNA was

measured between 2 and 3.5 hr after adding virus (Table 1).

At 35 C there was an approximately 55-fold increase in RNA production in infected cells compared to uninfected cells. At 39 to 40 C there was a 96% blockage of the synthesis of viral RNA. Addition of 15 or 25 μg of FPA per ml to cultures at 39 to 40 C did not enhance RNA synthesis but caused further inhibition. In other studies, FPA concentrations of 8 to 60 $\mu\text{g}/\text{ml}$ were tested, but increases in the synthesis of viral RNA were never observed.

DISCUSSION

After infection at 35 C, poliovirus induced the synthesis of RNA that eluted from MAK columns with 0.9 M NaCl. Over 95% of this RNA was sensitive to ribonuclease in 0.5 M NaCl buffer and precipitated with 2 M LiCl. Centrifugation in linear sucrose density gradients showed the RNA to have a sedimentation constant (S_{20}) of 34 ± 2 , comparable to RNA found in mature virus (1, 4).

The viral-induced RNA was synthesized between 1 and 2 hr after infection at 35 C and increased significantly thereafter (Fig. 1 to 3). At 39 to 40 C there was some inhibition of RNA synthesis between 1 and 2 hr (Fig. 1). Compared to the samples at 35 C, about 50 to 70% of the normal amount of RNA was produced. After 2 hr of infection at 39 to 40 C, a negligible degree of RNA synthesis occurred (Fig. 2 and 3).

The small amounts of RNA that were synthesized in the early stages of infection have been observed by others at 37 C (4). The chromatographic and sedimentation behavior of early RNA was identical to that of viral RNA, but the complementarity of this RNA was not established. It could be totally the "plus" type found in mature virus, complementary RNA, or a combination of these. At the present time, only the physical properties of the RNA are known.

Double-stranded RNA was not observed during the first 2 hr of infection. In a previous study, it was shown that double-stranded RNA of poliovirus eluted with 0.8 M NaCl (17). Denaturation during extraction at 60 C was not responsible for the failure to observe this type of RNA, because other studies indicate that double-stranded RNA is synthesized in measurable quantities only after the first 2 hr of viral replication (2, 17, 18). In all of these studies, extraction with hot phenol was a standard procedure. Cold phenol was not suitable because the efficiency of RNA extraction from HeLa cells is poor at 4 C. Since double-stranded RNA comprises about 1% of the virus-induced RNA, it was probably present during the early stages of infection, but the techniques available were not sufficiently sensitive for

FIG. 8. Sensitivity of poliovirus polymerase to heat (A) and manganese (B). An arithmetic plot of the sensitivities of control enzyme (X) and enzyme obtained from cells exposed to 25 μg of FPA per ml (●).

TABLE 1. Effect of FPA on the synthesis of poliovirus RNA at 35 C and 39 to 40 C

Sample	Counts per min per 100 μg of RNA
Uninfected cells at 35 C.....	384
Infected cells at 35 C.....	20,835
Infected cells at 35 C + 25 μg of FPA per ml.....	9,017
Infected cells at 39 to 40 C.....	1,286
Infected cells at 39 to 40 C + 15 μg of FPA per ml.....	883
Infected cells at 39 to 40 C + 25 μg of FPA per ml.....	834

its detection. This conclusion is based on the assumption that the proportion of double-stranded RNA to single-stranded RNA was the same during the early and late stages of infection. At the present time, there is no evidence to support or refute this hypothesis.

The absence of new RNA synthesis at 39 to 40 C after 2 hr of infection was not due to a combination of synthesis and rapid degradation of virus-induced RNA. If this occurred, the RNA that was synthesized between 1 and 2 hr should have been degraded after 2 hr. Figure 5a shows that this is not the case. The early RNA was metabolically stable at 39 to 40 C and remained unaltered. That is, early RNA was neither degraded nor converted to double-stranded RNA.

Recent studies by Lodish and Zinder (13) and Sugiyama and Nakada (20) showed strong regulation of protein synthesis among RNA phages. The coat protein of virus is capable of repressing translation of several viral cistrons. Experiments by Maizel and co-workers (15) and Holland (7) showed that regulatory mechanisms are also involved in controlling protein synthesis after poliovirus infection.

The data in Fig. 6 to 8 are in agreement with previous experiments (8) and show FPA to have a relatively selective effect on viral capsid protein. However, FPA did not reverse the inhibition of RNA synthesis resulting from incubation of cells at 39 to 40 C. The data suggest that either coat protein of poliovirus does not function as a repressor of the polymerase cistron or that FPA does not affect repressor activity. At the present time, a definite conclusion cannot be reached.

Another explanation for the defect in LSc virus growth can be obtained from other studies (7, 10, 15). During the growth of poliovirus, some of the virus-induced proteins undergo specific cleavage. Blockage of this process at 39 to 40 C could be responsible for inhibition of virus development. Jacobson and Baltimore (10) recently showed that FPA blocks cleavage of some virus-induced proteins. This could account for the inhibition of synthesis of viral RNA and viral polymerase by FPA at both 35 C and at 39 to 40 C (Fig. 6 and Table 1).

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