Nucleic Acid of Rubella Virus and Its Replication in Hamster Kidney Cells

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Received for publication 27 October 1969

Ribonucleic acid (RNA) has been isolated from partially purified rubella virus preparations and fractionated by rate zonal centrifugation in sucrose density gradients. The bulk of the RNA sedimented as a sharp band with a sedimentation coefficient of 38S. Rubella virus RNA appears to be single-stranded on the basis of its sensitivity to the degrading action of ribonuclease. Fractionation by precipitation with 1 M NaCl, followed by chromatography on cellulose columns, and by rate zonal centrifugation in sucrose density gradients of labeled RNA isolated from actinomycin D-treated and infected baby hamster kidney cells revealed the presence of the following virus-specific types of RNA: (i) single-stranded RNA with a heterogeneous sedimentation pattern, the 38S viral RNA becoming the predominant species only after long periods of labeling late after infection; (ii) double-stranded RNA with a sedimentation coefficient of 20S; (iii) RNA apparently composed of 20S double-stranded RNA and single-stranded branches. On the basis of their properties, the last two species were tentatively identified as the replicative form and the replicative intermediate of rubella virus RNA. Rubella virus RNA was infectious.

Although rubella virus has been the subject of intensive laboratory investigation, little has been known about the nucleic acid of the virus and its replication in infected cells. Two lines of evidence suggested that rubella virus is a ribonucleic acid (RNA) virus. Virus replication was not inhibited by deoxyribonucleic acid (DNA)-specific inhibitors (21, 23, 28), and labeled uridine was incorporated into material that co-sedimented in various gradients with the hemagglutination (HA) of the virus (5). Rubella virus was also observed to be similar morphologically to arboviruses (16).

It is shown in this study that rubella virus contains a single-stranded RNA with a sedimentation coefficient of 38S. The mode of replication of the viral RNA in BHK cells was also investigated. In addition to viral RNA, infected cells synthesized rubella virus-specific RNA with properties similar to replicative form (RF) and replicative intermediate (RI) RNA species isolated from cells infected with other RNA-containing viruses (1, 7, 8, 10, 11, 26, 33, 42). Studies with these other viruses indicated that the replication of single-stranded viral RNA is initiated by the synthesis of a complementary strand which forms, with the parental strand, a double-stranded, ribonuclease-resistant structure (RF).

The progeny RNA is then synthesized on the RF as single-stranded branches, base paired in the vicinity of their growing points to a complementary RNA template, the resulting structure (RI) being only partially refractory to the degrading action of ribonuclease.

MATERIALS AND METHODS

Cells. BHK-21/13S cells (37, 40), of baby hamster kidney (BHK) origin, were grown at 37°C in 2-liter roller bottles or roller tubes in BHK-21 medium (37) containing 10% inactivated (50°C for 30 min) fetal calf serum.

Virus. The RA27/3 strain of rubella virus (29), plaque purified and serially passaged in BHK cells at an input multiplicity of 1 plaque-forming unit (PFU) per cell, was used in all experiments. It was passaged no more than four times after plaque purification. Stock suspensions of extracellular virus contained an average of 10⁸ PFU per ml.

Virus assay. The assay of infectious virus by plaque technique (30) and the titration of viral hemagglutinin (12) were described previously.

Preparation of ³H- or ¹⁴C-uridine-labeled virus. Roller cultures of BHK cells were inoculated with 10 PFU of rubella virus per cell, and the virus was allowed to adsorb at 35°C. The time of virus addition was considered 0 hr of infection. After 2 hr of adsorption, the cells were washed with Eagle's basal medium and covered with BHK-21 medium without tryptose phosphate broth (50 ml per 10⁸ cells) containing ³H-uridine (2 µc/ml; specific activity, 25 c/mmole);

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New England Nuclear Corp., Boston, Mass.) and 2 to 5% fetal calf serum. [Serum used in these experiments was heated at 56 C for 30 min and freed from low-molecular-weight material by dialysis and of material of molecular weight greater than 200,000 daltons by filtration through a Diaflo XM100 membrane (Amicon, Lexington, Mass.).] Cultures were then incubated at 35 C. For some experiments, the virus was grown in cells treated between 8 and 13 hr postinfection with 1 μ g of actinomycin D per ml and labeled after the removal of the antibiotic with 0.25 μ Ci of uridine-2-¹⁴C per ml (53 mCi/mole, New England Nuclear Corp.). Extracellular virus was harvested at 36 to 48 hr postinfection. The yield of infectious virus and of viral hemagglutinin varied from 7×10^7 to 3×10^8 PFU per ml and 32 to 256 HA units per ml, respectively.

Concentration and purification of virus. Infectious tissue culture fluid was clarified by centrifugation at $3,000 \times g$ for 20 min. Tris(hydroxymethyl)aminomethane (Tris) and ethylenediaminetetraacetate (EDTA) were added to the supernatant fluid to 0.01 M concentration. Tris buffered the virus suspension at about pH 7.4, and EDTA bound Ca^{++} and Mg^{++} ions, dissociating possible complexes between serum components and virus particles (W. D. Sedwick, T. Furukawa, and S. A. Plotkin, *Bacteriol. Proc.*, p. 180, 1968). Virus (which does not pass through a Diaflo XM100 membrane) was concentrated by ultrafiltration, diluted 10-fold with 0.15 M NaCl, 0.05 M Tris-hydrochloride, 0.01 M EDTA (pH 7.8), and concentrated again. The dilution and concentration steps were repeated to ensure removal of material which could pass through the membrane. The 20-fold concentrated virus was then clarified by centrifugation at $10,000 \times g$ for 10 min.

A 5-ml amount of 25% (w/w) sucrose in 0.15 M NaCl, 0.05 M Tris-hydrochloride, and 0.001 M EDTA (pH 7.8; NTE buffer) was placed on top of 5 ml of 45% sucrose. About 20 ml of concentrated virus suspension was then layered on the 25% sucrose solution, and the contents of the tube were centrifuged in the SW 25.1 rotor of a Spinco centrifuge at $54,000 \times g$ at 4 C for 2 hr. The visible band at the interphase of the two sucrose solutions was collected and diluted with 0.15 M NaCl, 0.05 M Tris-hydrochloride, 0.001 M MgCl_2 (pH 7.8; NTM buffer) to 29% sucrose. Deoxyribonuclease (20 μ g/ml) free from ribonuclease (Worthington Biochemical Corp., Freehold, N.J.) and MgCl_2 to 3×10^{-3} M were added and the mixture was incubated at 22 C for 20 min. Portions (1-ml) of deoxyribonuclease-treated virus were then layered on a 10-ml, 30 to 45% linear gradient of sucrose in NTE buffer and centrifuged in the SW 40 rotor of a Spinco centrifuge at $192,000 \times g$ at 4 C for 3 hr. Fractions of 0.5 ml were collected and their hemagglutinin content was determined. Fractions corresponding to the peak of HA activity were pooled (1.5 to 2.0 ml) and used for RNA isolation. In some experiments, the virus was further purified by layering 1-ml samples of diluted virus suspension on 4.5-ml, 30 to 45% linear gradients of sucrose in NTE buffer and centrifuging in an SW 50 rotor of a Spinco centrifuge at $130,000 \times g$ at 4 C for 8 hr. The

virus peak was collected as in the previous gradient centrifugation.

Disruption of the virions and isolation of viral RNA.

RNA was released from purified virus by treatment with 1% sodium dodecylsulfate (SDS) at 22 C for 1 hr. Nonlabeled BHK-cell RNA (100 to 200 μ g) and NaCl to 1 M concentration were then added. The nucleic acid was precipitated by 2.5 volumes of ethanol at -20 C for 12 hr. In some experiments, the released viral RNA was deproteinized by two subsequent treatments with phenol (22 C, 10 min) and then precipitated by ethanol.

Isolation of RNA from infected and uninfected cells.

Cells were scraped off the glass surface, combined with the sediment from the clarification of the corresponding tissue culture fluid, washed three times with NTM buffer, and suspended in the same buffer to a concentration not exceeding 3×10^7 cells per ml. SDS was added to the suspension to 1% concentration, and the disrupted cells were shaken at 22 C for 10 min with an equal volume of 80% phenol containing 0.1% 8-hydroxyquinoline (19). The aqueous phase, separated by low-speed centrifugation, was extracted two more times with phenol. After adjusting the concentration of NaCl to 1 M in the aqueous phase, the nucleic acids were precipitated from this phase with ethanol. The precipitate was collected by low-speed centrifugation, washed with cold 70% ethanol, dried, and dissolved in NTM buffer. DNA was degraded by the addition of 20 μ g of deoxyribonuclease per ml free from ribonuclease and incubated at 22 C for 30 min. Pronase (Calbiochem, Los Angeles, Calif.) preincubated at 37 C for 30 min was then added (1 mg/ml), and the mixture was incubated for an additional 30 min at 37 C. The RNA was then treated once more with phenol and precipitated by ethanol. The precipitated RNA was collected by low-speed centrifugation and washed with cold 70% ethanol before further fractionation or analysis.

Fractionation of RNA. RF and transfer RNA were separated from single-stranded RNA and RI by precipitation of the latter with 1 M NaCl (3). RI was separated from single-stranded RNA by chromatography on a cellulose column (8) by using the modifications introduced by Bishop and Koch (4). To determine the proportion of RNA refractory to the action of ribonuclease, labeled RNA in 0.3 M NaCl, 0.05 M Tris-hydrochloride, 0.001 M MgCl_2 (pH 7.8; 2NTM buffer) was digested at 37 C for 30 min with 5 or 10 μ g of ribonuclease per ml (Worthington Biochemical Corp.) in the absence and presence of SDS, respectively. Samples containing 0.1% SDS were diluted 10-fold in 2NTM buffer before treatment with ribonuclease. The action of the enzyme was stopped by the addition of cold trichloroacetic acid, followed by the addition of 50 μ g of carrier yeast RNA. These conditions of ribonuclease treatment were found to reduce the acid-precipitable radioactivity derived from single-stranded RNA to background levels. Sucrose density gradients were sampled by inserting a tube to the bottom of the centrifuge tube and pumping its contents through the flow cell of a Gilford spectrophotometer to record the optical density at 260 nm and to collect the fractions.

Assay of infectious RNA. BHK cells were washed three times with NTM buffer and suspended at a concentration of 10^7 cells per ml in NTM buffer containing 500 μ g of diethylaminoethyl (DEAE)-dextran per ml (22, 38). RNA to be assayed was diluted 10-fold in NTM buffer containing DEAE-dextran, and 1 ml of each dilution was mixed with the same volume of cell suspension. After 20 min of incubation at 22 C, the cells were sedimented at $500 \times g$ for 5 min. The supernatant fluid was discarded and the cells were suspended in 1 ml of BHK-21 medium supplemented with 2% inactivated fetal calf serum. The inoculated cells were then processed in the same manner as cells infected with virus.

Measurement of radioactivity. For determination of total radioactivity, 0.02- to 0.10-ml portions of the samples were dried directly on glass-fiber filters (934 AH, 2.4 cm, Reeve Angel, Clifton, N.J.). Acid-precipitable radioactivity was determined after the addition of 50 μ g of carrier yeast RNA and of cold trichloroacetic acid to a concentration of 5 or 10% in the absence and in the presence of SDS, respectively. After 1 hr at 0 C, precipitates were collected on membrane filters (B-6, Bac-T-Flex, Schleicher and Schuell, Keene, N.H.), washed with cold 5% trichloroacetic acid, and dried. The filters were then placed in 5 ml of Liquifluor (New England Nuclear Corp.) in toluene, and the radioactivity was measured in a Packard liquid scintillation spectrometer.

Chemical analysis. Protein concentrations were determined by the method of Lowry et al. (20) by using bovine serum albumin as standard.

RESULTS

Purification of rubella virus. In the procedure used for the purification of rubella virus, ultrafiltration reduced the protein content of the crude virus preparation by 98% on the average. In most experiments, 85 to 100% of the infectivity and HA activity was recovered in the concentrate. The filtrates were always free from virus. Sedimentation of the virus on the sucrose cushion did not cause significant losses in biological activities, although 93% of the proteins and 90% of the radioactive uridine layered on the sucrose solution were removed during this step. About 60% of the input HA activity and infectivity was recovered in the virus band after centrifugation in sucrose density gradients. The largest sharp peak of radioactivity always coincided with the peaks of infectivity and HA activity. The final preparation contained 15 to 50% of the HA units and 10 to 30% of the PFU present in the original, crude virus preparation. Virus purified by this procedure was still contaminated with host-cell components. This was revealed during further purification of some virus preparations by centrifuging to equilibrium in sucrose density gradients (Fig. 1) and by analysis of the RNA released from purified virus (*see below*).

Nucleic acid of rubella virus. Labeled RNA was

FIG. 1. Equilibrium density gradient centrifugation of rubella virus. A 1-ml amount of concentrated and partially purified virus was layered on a 4.5 ml, 30 to 45% linear gradient of sucrose in NTE buffer and centrifuged at $130,000 \times g$ for 8 hr at 4 C. Fractions were collected and analyzed for total radioactivity and HA activity. The density at the virus peak was 1.185 g/cm³. Symbols: ○, radioactivity of ³H-uridine; ●, HA activity.

released from purified virions by treatment with SDS or by extraction with SDS and phenol. It was then analyzed by rate zonal centrifugation in sucrose density gradients (Fig. 2). The main component of virus RNA sedimented as a sharp band with a sedimentation coefficient of 38S relative to BHK-cell ribosomal RNA (24). In some preparations, minor components having sedimentation properties similar to those of BHK-cell ribosomal RNA (28S and 18S) were present. They were absent, or markedly reduced, however, in RNA preparations isolated from labeled virions grown in cells treated with actinomycin D (Fig. 3). All preparations contained a variable proportion of another minor component with a sedimentation coefficient of about 25S. The origin of this component was not further investigated. The viral RNA, as well as other components isolated from partially purified virions, is single-stranded as indicated by its sensitivity to the degrading action of ribonuclease (Fig. 3).

Effect of actinomycin D on the synthesis of cellular RNA and the replication of rubella virus. No effect on the synthesis of cellular RNA was detected after infection of BHK cells with rubella virus (23). Therefore, to label preferentially the virus-specific RNA, it was necessary to suppress the synthesis of host cell RNA by actinomycin

FIG. 2. Rate zonal centrifugation in a sucrose density gradient of RNA isolated from rubella virus. RNA was released by SDS from 3,000 HA units of partially purified and ^3H -uridine-labeled rubella virus, mixed with 100 μg of carrier BHK-cell RNA, concentrated by precipitation with ethanol to 1 ml, and layered on a 29 ml, 10 to 35% linear gradient of sucrose in NTE buffer containing 0.1% SDS. The contents of the tube were centrifuged at $59,000 \times g$ for 9 hr at 20 C. Fractions were collected and the absorbance at 260 nm (solid line) and the acid-precipitable radioactivity (interrupted line) were determined.

FIG. 3. Rate zonal centrifugation in a sucrose density gradient of RNA isolated from partially purified rubella virus grown in actinomycin D-treated cells. RNA was released from 2,880 HA units of ^{14}C -uridine-labeled virus, mixed with 200 μg of carrier BHK-cell RNA, concentrated, and fractionated by density gradient centrifugation as described in Fig. 2. Fractions were collected, and the absorbance at 260 nm and the acid-precipitable (○) and ribonuclease-resistant (●) radioactivities were determined.

D. BHK cells had to be pretreated for several hours with the drug to inhibit efficiently the transcription of the cell genome. More than 95% inhibition of cellular RNA synthesis was observed after a 4-hr treatment of BHK cells with 1 to 2 μg of actinomycin D per ml (Table 1). Exposure of the cells to higher concentrations of actinomycin D (5 or 10 μg per ml) for a shorter time (1 or 2 hr) caused a greater toxic reaction, manifested by rounding and detachment of the cells from the glass surface, than treatment of the cultures with lower concentrations of the drug (1 or 2 μg per ml) for longer time periods (4 or 8 hr). Inhibition of RNA synthesis by actinomycin D in BHK cells was irreversible, even after replacing the medium containing the drug with fresh medium, as was observed with other cells (36).

Newly synthesized rubella virus was first detected in untreated and actinomycin D-treated cultures at 10 to 12 hr after infection (Fig. 4). The addition of actinomycin D at this time slightly increased the rate of virus replication and did not affect the maximum yield of virus. A 4-hr exposure of cultures to the drug before infection had no effect on the initial rate of virus replication, but by 36 hr postinfection, i.e., 40 hr after actinomycin D treatment, the virus yield was reduced 10-fold in comparison with the

TABLE 1. Inhibition of RNA synthesis in noninfected BHK cells by actinomycin D^a

Actinomycin D concn ($\mu\text{g}/\text{ml}$ of medium)	Duration of treatment (hr)	Counts per min per μg of RNA	Inhibition of ^3H -uridine uptake (%)
0		1,695 ^b	0
0.5	1	334	80.3
	2.5	214	87.4
	4	131	92.3
1.0	1	212	87.5
	2.5	94	94.5
	4	53	96.9
2.0	1	210	87.6
	2.5	92	94.6
	4	42	97.5

^a Monolayer cultures containing 6×10^6 cells were pretreated for 1 to 4 hr with various concentrations of actinomycin D in 2 ml of medium supplemented with 2% inactivated fetal calf serum. The actinomycin D-containing medium was then removed, and the cultures were covered with 2 ml of fresh medium containing 2 μC of ^3H -uridine. After the labeling, the cells were harvested; the ribonucleotides were isolated and their concentration was determined on the basis of absorbance at 260 nm and related to the radioactivity of incorporated ^3H -uridine.

^b Average of two determinations (1,630 and 1,760 counts per min per μg of RNA).

FIG. 4. Effect of actinomycin D on the replication of rubella virus in BHK cells. Confluent roller cultures containing 10^7 cells were infected with 10 PFU of virus per cell. Some cultures were treated for 4 hr before infection with 2 ml of medium containing 0.5 μ g (●) or 2 μ g (○) of actinomycin D per ml (panel A); others were treated with 0.5 μ g (▲) or 2 μ g (△) of actinomycin D per ml between 11 and 15 hr postinfection (panel B). One set of cultures was treated continuously with 0.5 μ g of actinomycin D per ml from 4 hr before infection throughout the growth cycle of the virus (□; panel A). All cultures were maintained before and after actinomycin D-treatment with 2 ml of BHK-21 medium supplemented with 2% inactivated fetal calf serum. Control cultures (■) were infected similarly but were not treated with the drug (panel B). Cultures were harvested at intervals after infection and subjected to sonic treatment for 2 min at 10 kc. The released virus was assayed by plaque technique.

untreated control. The presence of actinomycin D throughout the experiment slightly decreased the rate of virus replication and also reduced the virus yield. The decreased virus yield observed after exposure of the cells to actinomycin D before infection or early after infection was probably caused by the toxic effect of the drug on the cells. Indeed, staining with 0.25% trypan blue showed that the number of viable cells in cultures exposed for 30 hr to actinomycin D was 20 to 40% of the untreated control.

Synthesis of virus-specific RNA. The time

dependence of synthesis of total and ribonuclease-resistant virus-specific RNA is shown in Fig. 5. Synthesis of virus-specific RNA was not detected earlier than 8 hr postinfection. Later, the rate of total and ribonuclease-resistant virus-specific RNA increased up to 38 hr postinfection, approximately in parallel with the rate of replication of infectious virus.

Characterization of virus-specific RNA accumulated in the cells during infection. To characterize the virus-specific RNA accumulated in the cells during the replication cycle of the virus, RNA isolated from actinomycin D-treated cells at 35 hr after infection was fractionated with 1 M NaCl and analyzed by sucrose density gradient centrifugation (Fig. 6). The predominant compo-

FIG. 5. Time dependence of virus-specific RNA synthesis. Roller cultures containing 1.5×10^8 cells were infected with 10 PFU of virus per cell or mock-infected with a corresponding amount of medium. Actinomycin D (40 μ g in 20 ml of medium supplemented with 2% dialyzed and inactivated fetal calf serum) was added to infected or mock-infected cells at intervals for 8 hr prior to labeling for 6 hr with 50 μ Ci of 3 H-uridine in 10 ml of medium. The labeled cells were then harvested and the RNA was extracted with SDS and phenol. The acid-precipitable radioactivity before and after ribonuclease digestion was determined and normalized to the same amount of ribosomal RNA. Symbols: ○, total acid-precipitable radioactivity incorporated into the RNA of infected cells; △, total acid-precipitable radioactivity incorporated into the RNA of mock-infected cells; ●, acid-precipitable radioactivity incorporated into ribonuclease-resistant RNA of infected cells; ▲, acid-precipitable radioactivity incorporated into ribonuclease-resistant RNA of mock-infected cells.

FIG. 6. Analysis by rate zonal centrifugation in sucrose density gradients of virus-specific RNA accumulated in the cells during infection. A total of 3×10^6 cells in roller cultures was infected with 10 PFU of virus per cell and incubated in 420 ml of medium containing 2% dialyzed and inactivated fetal calf serum. Actinomycin D ($1 \mu\text{g/ml}$) was added 8 hr after infection. The medium was removed 13 hr postinfection and replaced with the same volume of fresh medium containing $840 \mu\text{C}$ of ^3H -uridine. The cells were harvested at 35 hr postinfection. RNA was isolated by treatment with SDS and phenol and fractionated by precipitation with 1 M NaCl. Samples of the precipitable (panel A) and nonprecipitable (panel B) RNA were layered on 36 ml, 10 to 35% linear gradients of sucrose in NTE buffer containing 0.1% SDS and centrifuged at $88,000 \times g$ for 11 hr at 20°C . Fractions were collected and their absorbance at 260 nm was determined. Odd-numbered fractions were assayed for total acid-precipitable radioactivity (○). Even-numbered fractions were analyzed for acid-precipitable radioactivity resistant to ribonuclease (●). RNA sedimented to the

nents of labeled RNA precipitable by 1 M NaCl (Fig. 6, panel A) had sedimentation coefficients of 38S, 34S, and 23S. The possibility that additional components of virus-specific RNA are obscured by incorporation into the RNA of the host cell cannot be, however, excluded. The 38S RNA most likely represented viral RNA. Degradation of single-stranded RNA by ribonuclease revealed a component resistant to the action of the enzyme and sedimenting principally at 21S. The RNA soluble in 1 M NaCl (Fig. 6, panel B) contained a 20S ribonuclease-resistant component (RF) which was absent from uninfected controls. Under the conditions of labeling and actinomycin D treatment used in this experiment, RNA soluble in 1 M NaCl contained a relatively small amount of RF (compare with Fig. 8, panel A). The sedimentation pattern of labeled RNA fraction derived from similarly treated uninfected cells and insoluble in 1 M NaCl is shown in Fig. 6 (panel C).

A sample of RNA from infected cells was fractionated further by cellulose column chromatography (Fig. 7, panels A and B). RNA insoluble in 1 M NaCl was loaded on a cellulose column in a buffer solution containing ethanol. The single-stranded species of RNA in this solvent are not adsorbed to the cellulose. Buffer solution without ethanol was then used to elute the RNA which had a higher degree of secondary structure than single-stranded RNA. This RNA was assumed to be RI consisting of single strands attached to double-stranded RNA. By this criterion, RI represented 12% of the virus-specific RNA insoluble in 1 M NaCl. Single-stranded RNA species freed from RI were analyzed by rate zonal centrifugation in sucrose density gradient (Fig. 7, panel C). The 38S and 34S components were again present in the same proportion as in the total RNA insoluble in 1 M NaCl (compare with Fig. 6). The 23S RNA component clearly discernible before the chromatography (Fig. 6) was reduced and a 25S RNA component was revealed. The single-stranded RNA fraction did not contain detectable amounts of ribonuclease-resistant RNA.

A large portion of RI purified by repeated

bottom of the gradient was also resuspended and its acid-precipitable radioactivity was determined before and after treatment with ribonuclease. RNA from similarly treated but uninfected cells (panel C) was fractionated and analyzed similarly. The sedimentation pattern of the fraction soluble in 1 M NaCl is not shown, however, since it was similar to that of the corresponding RNA from infected cells (panel B), except for the absence of the small amount of 20S component resistant to the action of ribonuclease.

column chromatography represented double-stranded RNA, since 61% of the acid-precipitable radioactivity contained in the RI fraction was refractory to the action of ribonuclease. The bulk of RI had a sedimentation coefficient of 21S (Fig. 7, panel D). The band of RI, as well as that

of its ribonuclease-resistant core, was, however, skewed toward the bottom of the gradient. The double-stranded core of RI, isolated after treatment of RI at 37 C for 30 min with 2 μ g of ribonuclease per ml, sedimented as a single 19S band skewed toward the top of the gradient (not shown in Fig. 7).

Fractionation and properties of virus-specific RNA that was synthesized at the time of its maximum rate of replication. The proportion of various species of virus-specific RNA accumulating in cells during the whole virus replication cycle is determined by the rate of their synthesis, turnover, release, and degradation. In an attempt to characterize the components of virus-specific RNA synthesized during the time interval when the rate of their synthesis was maximal, actinomycin D-treated cells were labeled with 3 H-uridine between 26 and 32 hr postinfection, and the RNA isolated was analyzed as described in the previous paragraph (Fig. 8). An appreciable amount of RF soluble in 1 M NaCl and almost completely resistant to ribonuclease was present in the preparation. The bulk of the RF sedimented at 20S, but the band was skewed toward the top of the gradient. The radioactivity incorporated into the virus-specific RNA precipitable by 1 M NaCl was heterogeneously distributed throughout the gradient. A 22S component became, however, clearly discernible. About 10% of the labeled RNA precipitable by 1 M NaCl sedimented to the bottom of the gradient. The sedimentation pattern of RNA fractions isolated from similarly treated uninfected cells is also shown in Fig. 8.

To resolve the complex sedimentation pattern of RNA precipitable by 1 M NaCl, RI and single-stranded RNA were separated by chromatography on a cellulose column and analyzed by sucrose density gradient centrifugation (Fig. 9). A significant portion of the RNA derived from cells labeled between 26 and 32 hr after infection sedimented faster than 38S viral RNA. Most of this heavy RNA was separated from single-stranded RNA by cellulose column chromatography (see Fig. 8, panel A, and Fig. 9, panel B). This indicates that the bulk of the RNA, sedimenting faster than 38S, was either RI or aggregated RNA with a high degree of secondary structure. The sedimentation pattern of ribosomal RNA was not affected by the column fractionation procedures (Fig. 9, panel A). The bulk of the 23S RNA was also absent from the single-stranded RNA fraction. The single-stranded RNA recovered in this region of the gradient was probably of cellular origin, as indicated by a parallel control experiment (Fig. 8, panel B). The sedimentation pattern of RI synthesized

FIG. 7. Fractionation by cellulose column chromatography and rate zonal centrifugation in sucrose density gradient of RNA accumulated in the infected cells and insoluble in 1 M NaCl. RNA, described in Fig. 6, in 40 ml of 0.1 M NaCl, 0.001 M EDTA, 0.001 M Tris-hydrochloride (pH 7.4), containing 15% ethanol (STE-15% EtOH), was loaded on a column (1.5 by 28 cm) of cellulose equilibrated with STE-35% EtOH. To elute single-stranded RNA, the column was exhaustively washed with STE-15% EtOH at a flow rate of 1 ml/min. RI was eluted from the column with STE buffer (panel A). RI was then adjusted to STE-35% and was rechromatographed in a similar manner (panel B). The single-stranded RNA eluted from the column by STE-15% EtOH was concentrated by precipitation with ethanol and analyzed by rate zonal centrifugation in sucrose density gradient (panel C) as described in Fig. 6. The RI was mixed with 500 μ g of carrier BHK-cell RNA, concentrated and analyzed similarly (panel D). Panels A and B: $\bullet$, total radioactivity per fraction. Panels C and D: $\circ$, acid-precipitable radioactivity; $\bullet$, acid-precipitable radioactivity resistant to ribonuclease.

from 26 to 32 hr postinfection was more heterogeneous (Fig. 9, panel B) than that of RI isolated from cells labeled during the whole virus replication cycle (Fig. 7, panel D). The RI fraction repre-

FIG. 8. Sucrose density gradient analysis of virus-specific RNA synthesized in the cells between 26 and 32 hr after infection. Roller culture containing 1.5×10^8 cells was infected with 10 PFU of rubella virus per cell. The cells were treated between 18 and 26 hr after infection with 20 ml of medium containing 2 μ g of actinomycin D per ml. The drug was then removed and 10 ml of medium containing 50 μ Ci 3 H-uridine was added. The cells were labeled for 6 hr. Uninfected cells were treated and labeled similarly. RNA was isolated, fractionated by 1 M NaCl, and analyzed by rate zonal centrifugation in sucrose density gradient as described in Fig. 6. Fractions were collected and analyzed as described in Fig. 3. Symbols: $\circ$, acid-precipitable radioactivity insoluble in 1 M NaCl; $\bullet$, acid-precipitable radioactivity soluble in 1 M NaCl; Δ , acid-precipitable radioactivity incorporated into RNA soluble in 1 M NaCl and resistant to the action of ribonuclease. RNA sedimented to the bottom of the gradients A and B from the total RNA insoluble in 1 M NaCl contained 1,085 and 931 counts/min, respectively. The radioactivity of the RNA samples isolated from infected and mock-infected cells was normalized to the same amount of ribosomal RNA.

FIG. 9. Sucrose density gradient patterns of RNA species synthesized in infected and actinomycin D-treated cell cultures between 26 and 32 hr postinfection and prefractionated by cellulose column chromatography. Cells were infected, treated with actinomycin D, and labeled as described in Fig. 8. RNA was isolated from the cells and was fractionated by 1 M NaCl and by chromatography on a cellulose column (see Fig. 7). The individual RNA components were then centrifuged in a sucrose density gradient (panel B) as described in Fig. 6. The tissue culture fluid was clarified by centrifugation at $10,000 \times g$ at 4 C for 10 min and then centrifuged at $50,000 \times g$ at 4 C for 1 hr. RNA was isolated from the sediment and analyzed by centrifugation in sucrose density gradients (panel C), as described in Fig. 2. Panel A: solid line, sedimentation patterns of ribosomal RNA before cellulose column chromatography; interrupted line, the same after the chromatography. Panel B: $\circ$, acid-precipitable radioactivity in RNA insoluble in 1 M NaCl; Δ , acid-precipitable radioactivity incorporated into single-stranded RNA isolated by cellulose column chromatography; $\blacktriangle$, acid-precipitable radioactivity incorporated into RI isolated by cellulose column chromatography; $\bullet$, acid-precipitable radioactivity incorporated into the ribonuclease-resistant portion of total RNA insoluble in 1 M NaCl; $\square$, acid-precipitable radioactivity incorporated into the ribonuclease-resistant core of RI. Panel C: $\circ$, acid-precipitable radioactivity; $\bullet$, acid-precipitable radioactivity after treatment with ribonuclease.

sented 53% of the total virus-specific RNA. The expected peak of 21S RI component was clearly discernible.

Viral RNA sedimenting at 38S did not appear to accumulate in cells between 26 and 32 hr postinfection (Fig. 9, panel B). Thus, the viral RNA was either rapidly incorporated into virions and released into the medium or was not the predominant RNA species synthesized during this time interval. The following experiment was carried out to prove one of these alternatives. Virions were sedimented by high-speed centrifugation from the tissue culture fluid of actinomycin D-treated cultures, which were labeled with ^3H -uridine between 26 and 32 hr postinfection. RNA was then extracted from the resuspended sediment and analyzed by sucrose density gradient centrifugation (Fig. 9, panel C). The recovered amount of extracellular 38S viral RNA was similar to that of intracellular viral RNA. The sedimentation pattern was similar to that observed with RNA isolated from partially purified virus, but a small amount of ribonuclease-resistant RNA sedimenting at 20S was detected.

To determine which RNA species first incorporated the labeled precursor during the period when virus-specific RNA was synthesized at a maximum rate, cells treated with actinomycin D were exposed to ^3H -uridine for 30 and 90 min, at 26 hr postinfection. The RNA was then extracted, fractionated by treatment with 1 M NaCl, and analyzed (Fig. 10). The sedimentation pattern of RNA insoluble in 1 M NaCl was heterogeneous, but most of the ^3H -uridine was incorporated into partially ribonuclease-resistant components sedimenting at 21S to about 33S. The ribonuclease-resistant portion of the RI represented 26 and 28% of the total virus-specific RNA after 30 min and 90 min of labeling, respectively. In the case of the 30-min labeling period, the amount of ribonuclease-resistant RNA in the fraction precipitable by 1 M NaCl was determined on a portion of the sample before centrifugation. After 30 and 90 min of exposure of the infected cells to ^3H -uridine, 9 and 10%, respectively, of the total radioactivity of the virus-specific RNA were recovered in the RF fraction.

Infectivity of RNA isolated from rubella virus or from rubella virus-infected cells. The infectivity of rubella virus RNA assayed in suspension cultures was extremely low. An average of 10 plaques was obtained after inoculation of 38S viral RNA isolated by sucrose density gradient centrifugation and derived from 10^9 PFU of partially purified virus. No infectivity was observed after inoculation of similar amounts of viral RNA pretreated in 2NTM buffer solution at 37 C for 15 min with 5 μg of ribonuclease

FIG. 10. Sucrose density gradient patterns of RNA synthesized in actinomycin D-treated BHK cells during a 30- and 90-min exposure to radioactive precursor at 26 hr postinfection. Roller cultures containing 1.5×10^8 cells were infected with 10^6 PFU of rubella virus per cell and treated with 80 μg of actinomycin D in 40 ml of medium from 18 to 26 hr postinfection. Actinomycin D was then removed and the cells were exposed for 30 (panel A) or 90 min (panel B) to 10 ml of medium containing 50 μC of ^3H -uridine. Noninfected cells were treated with actinomycin D and labeled for 90 min similarly (panel C). RNA was extracted from all cultures, precipitated by 1 M NaCl, and analyzed by sucrose density gradient centrifugation as described in Fig. 6. Panel A: $\circ$, acid-precipitable radioactivity of RNA insoluble in 1 M NaCl; $\bullet$, acid-precipitable radioactivity of RNA soluble in 1 M NaCl. Panel B: $\circ$, acid-precipitable radioactivity of RNA insoluble in 1 M NaCl; $\bullet$, acid-precipitable radioactivity of RNA insoluble in 1 M NaCl and resistant to the action of ribonuclease (enzyme treatment performed after gradient centrifugation); $\square$, acid-precipitable radioactivity of RNA soluble in 1 M NaCl. Panel C: $\circ$, acid-precipitable radioactivity of RNA insoluble in 1 M NaCl.

per ml. When RNA was extracted from 10^8 rubella virus-infected cells 32 hr postinfection, the preparation contained 10^8 to 2×10^8 PFU. The infectivity of the preparation was reduced 5- to 10-fold after treatment with ribonuclease. After fractionation of RNA by 1 M NaCl, 10 to 20% of the infectivity was found to be associated with the RNA fraction soluble in 1 M NaCl and was not affected by treatment with ribonuclease.

The material from the area of the plaques induced by infectious RNA was collected and inoculated into BHK cell cultures. The virus progeny was neutralized by anti-rubella sera to the same extent as the original virus.

DISCUSSION

The results of the present study have shown that rubella virus contains single-stranded RNA and that this RNA is infectious. Using Spirin's formula (34), the molecular weight of the 38S viral RNA can be estimated to be 3.2×10^6 daltons. In addition to the 38S viral RNA, a ribonuclease-sensitive component with a sedimentation coefficient of about 25S was always present in RNA preparations isolated from partially purified virions. This RNA was not of cellular origin, because similar amounts of this component were present in RNA preparations derived from virions propagated in actinomycin D-treated cells. It may, however, represent the genome of incomplete, defective virus particles.

The yield of rubella virus in BHK cells is relatively high (40). Therefore, they seemed to be a suitable host for studying the replication of rubella virus. It was difficult, however, to establish one-step growth conditions in rubella virus-infected BHK cells. Infectious center assay showed that, although more than 50% of the cells were productively infected at 36 hr post-infection, often less than 10% of the cells were infected at the end of the adsorption period (16; W. D. Sedwick, *unpublished data*). Unexpectedly, the efficiency of infection was essentially independent of the input multiplicity of infection in the range of 3 to 100 PFU of virus per cell (W. D. Sedwick, *unpublished data*). At the present time, the replication of rubella virus and the synthesis of virus-specific products must be considered as occurring in several cycles. Therefore, the sequence of events in the replication of rubella virus RNA and the early and late functions of the viral genome could not be determined. Interferon probably did not play a significant role in the asynchrony of infection, because the efficiency of infection of cells treated with actinomycin D was essentially the same as that of untreated cells. Other possible explanations, such as interference by noninfectious virions with the replication of infectious rubella virus and the dependence of the initiation of infection on the cell replication cycle, should, therefore, be investigated.

The assumption was made throughout the present study that the fraction of cellular RNA synthesized, in the presence of actinomycin D, is the same in uninfected and infected cells. This

assumption was based on the observation that cellular RNA synthesis was not affected by infection of untreated cells with rubella virus (23). Nevertheless, the possibility that cellular RNA synthesis may be different in uninfected and infected cells that were treated with actinomycin D should be kept in mind in the quantitative interpretation of the experiments on virus-specific RNA synthesis. Infected cells treated with actinomycin D incorporated only three to four times more ^3H -uridine than uninfected actinomycin D-treated cells.

Species of virus-specific RNA with various degrees of secondary structure, i.e., single-stranded RNA, double-stranded RNA, and RNA with both double- and single-stranded characteristics, were detected in actinomycin D-treated and rubella virus-infected cells. The abundant evidence for the presence of RF and RI in cells infected with RNA-containing viruses (1, 7, 8, 10, 11, 26, 35, 42), along with the characterization of their properties by many techniques (4), made it possible to identify similar RNA components in rubella virus-infected cells. As in all other RNA virus replicating systems studied, the actual RF and RI RNA structures as isolated from the cell may be different from the functional replicative structures in situ, although it is generally believed that they are direct derivatives of true replicative intermediates (33, 42). One of the components of virus-specific, single-stranded RNA found in infected cells was the 38S infectious viral RNA. Single-stranded RNA sedimenting at 26S was also virus-specific because it was not found in noninfected cells. Its function, however, remains obscure. The bulk of RF of rubella virus RNA sedimented at 20S. The RF band, however, always showed a trailing shoulder at about 15S. A similar heterogeneity was observed in the sedimentation pattern of RF from MS-2 bacteriophage-infected cells (17). The function and origin of this "light" double-stranded RNA are unknown. The results of the pulse-labeling experiments are consistent with the functioning of double-stranded, rubella virus-specific RNA as the site of viral RNA synthesis.

The observation that cells treated with actinomycin D from 8 to 35 hr postinfection and labeled with ^3H -uridine from 13 to 35 hr contain relatively small amounts of RF can be explained in the following way. The toxicity of actinomycin D is increasingly manifested with time. Therefore, after prolonged exposure to actinomycin D, infected cells synthesize markedly less virus and virus-specific RNA when compared with cells treated with actinomycin D for a short time and labeled in the same interval. RF of other RNA-

containing viruses is not conserved in the infected cells (18), and the same seems to be true for rubella virus. Therefore, the relative amount of RF must be reduced in cells in which the synthesis of virus and virus-specific RNA is depressed. This explanation is also supported by the fact that the RI fraction isolated from such cells does not contain the heavy components seen in similar RNA fractions derived from cells treated with actinomycin D for a shorter time.

Electron microscopic examinations have shown that rubella virus particles are morphologically similar to many arboviruses. They are roughly spherical with a diameter of 50 to 70 nm and with a 30-nm, electron-dense core (2, 6, 14-16, 27, 31, 41). A study of antigenic cross-reaction between rubella virus and arboviruses showed no relationship (25), and no evidence linking rubella virus with a vertebrate-arthropod vector cycle has been found. The results of the present study, however, strongly indicate similarities in the mode of replication of the RNA components of these viruses. The sedimentation coefficient of infectious, single-stranded RNA from group A arboviruses varies between 40S and 42S (11, 35). Cells infected with arboviruses synthesize a single-stranded, virus-specific 26S RNA and a double-stranded RNA sedimenting at 20S (11, 32). Arbovirus 42S RNA is also rapidly released from the cell in virus particles (36). The sedimentable particulate material containing ribonuclease-resistant RNA and released by rubella virus-infected cells may be similar to the structures found in the cytoplasm of cells infected with arboviruses and other small RNA viruses (9, 13). Rubella virus may differ, however, from arboviruses in one respect. The NaCl-soluble fraction contains infectivity, whereas the double-stranded RNA from arbovirus-infected cells is not infectious (11). In this respect, rubella virus may be more similar to poliovirus (4).

Thus far the infectivity assay for rubella virus RNA and for its RF is inefficient in comparison with the assay methods for infectious RNA of other viruses. More studies on the optimal conditions for assay are needed before infectivity can be used as a reliable tool for investigation of the properties of various species of rubella virus-specific RNA.

Studies of other investigators (T. Hovi, and A. Vaheri, *in preparation*; K. Wong and T. Merigan, *personal communication*) on the properties of rubella virus RNA and the mode of its replication produced results consistent with those described in this paper. Hovi and Vaheri were unable, however, to demonstrate infectivity of viral RNA in monolayer cultures of BHK-21/WI-2 cells.

ACKNOWLEDGMENTS

One of us (W. D. S.) thanks Stanley Plotkin for his support throughout the course of this work. The technical assistance of Lois Colbert is gratefully acknowledged.

This investigation was supported by Public Health Service training grant T01-GM 00142 from the National Institute of General Medical Sciences; funds from Smith, Kline and French Co.; the Pennsylvania Department of Health contract 69-593-1, ME-561 from the Commonwealth of Pennsylvania; and Public Health Service grant R01-CA 10594 from the National Cancer Institute.

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