Rubella Virus-specific Ribonucleic Acids in Infected BHK 21 Cells

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SUMMARY

The nucleic acid of rubella virus was shown to be RNA by the incorporation of [3H]-uridine into virus particles, and by the presence of ribonuclease-sensitive material, sedimenting mainly at 38 to 40 s, in sodium dodecyl sulphate extracts of purified virus. There were at least two virus-specific RNAs in cytoplasmic extracts of BHK 21 cells infected with rubella virus. One, possibly the structural RNA of the virus, was sensitive to ribonuclease and sedimented at 38 to 40 s like the RNA extracted from virus particles. The other had a heterogeneous sedimentation pattern with main peak at 20 s. It was partially resistant to ribonuclease and might be the replicative intermediate form of the RNA of rubella virus.

INTRODUCTION

The evidence that rubella virus is an RNA virus is indirect: thymidine analogues do not affect its multiplication (Maassab & Cochran, 1964), and actinomycin D has little or no inhibitory effect on its growth in tissue culture (Prinzie, 1964; Maes et al. 1966; Vaheeri, Plotkin & Sedwick, 1967; Woods & Robbins, 1968). Morphologically rubella virus resembles in some respects certain members of the arbo and leucosis virus groups (Holmes & Warburton, 1967; von Bonsdorff & Vaheeri, 1969). Information on the properties of rubella virus nucleic acid and on its metabolism in infected cells should clarify its classification.

METHODS

Infection and labelling of cells. Baby hamster kidney cells (BHK 21) and rubella virus (strain RA27/3) adapted to these cells were used. The history and cultivation of cells and virus and the plaque assay in BHK 21/WI-2 cells were described by Vaheeri et al. (1969). Usually 1.5 x 10^8 cells in suspension were infected with rubella virus (3 to 30 p.f.u. per cell) and incubated at 34°. The culture medium was BHK 21-medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% tryptose phosphate broth and 5% heat-inactivated calf serum. At 14 to 21 hr after infection 0.3 μg./ml. of actinomycin D (given by Merck, Sharp and Dohme, West Point, Pa.) was added. One hour later 1 μc/ml. of [3H]-uridine of specific activity 23 or 27.7 c/m-mole (Radiochemical Centre, Amersham, England) was added. Incorporation was terminated 1 ½ to 3 ½ hr later. Labelled virus was prepared by a similar infection procedure, with the addition of 4 μc/ml. of [3H]-uridine at 18 hr and a published purification method (Vaheeri et al. 1969).
Extraction of cytoplasmic RNA. The extraction procedure was a modification of the method used by Kääriäinen & Gomatos (1969). The cells were collected by centrifugation, washed in cold phosphate-buffered saline and suspended at about 5 × 10⁷ cells/ml in cold reticulocyte swelling buffer (0.01 M-tris-HCl, pH 7.4, 0.01 M-KCl and 0.0015 M-MgCl₂) containing potassium polyvinylsulphate 60 μg./ml. The cells were allowed to swell for a few minutes at 0 ° and then disrupted with a Dounce homogenizer. The homogenate was centrifuged at 500 g for 5 min. to give a nuclear pellet with most of the cytoplasm in the supernatant fluid. The cytoplasmic fraction (3.5 ml.) was adjusted to 1 mM-EDTA and was treated with 1.45% sodium dodecyl sulphate (crystallized according to Mandel, 1964) for 30 to 120 min. at room temperature.

RNA extraction from virus. Rubella virus labelled with [³H]-uridine was purified as described by Vaheri et al. (1969). The purified virus in phosphate-buffered saline was adjusted to 1 mM of EDTA and then incubated for 20 min. at 20 ° with 2% sodium dodecyl sulphate. The extract was then either directly analysed in a sucrose gradient or further extracted with phenol (Gierer & Schramm, 1956).

Analysis of extracts. A 15% to 30% linear gradient was made of ribonuclease-free sucrose (Sucrose cryst., density-gradient grade, Mann Research Laboratories, N.Y.) in reticulocyte swelling buffer. Two and a half ml. of RNA-extract were layered on this gradient and centrifuged for 16 hr at 52,000 g at 20 ° in rotor SW27 of a Spinco L2-50 ultracentrifuge. Fractions of 1 ml. were collected through the bottom of the tube. Extinction at 260 nm. was estimated in an ‘Opton’ spectrophotometer. Acid-insoluble radioactivity was measured by precipitation of RNA with 5% trichloracetic acid. The precipitates were collected on Millipore filter discs with 0.45 nm. pores. The discs were dried and immersed in scintillation fluid (50 mg./l. POPOP and 4 g./l. PPO into luene) and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3003.

To test ribonuclease sensitivity the samples were incubated at 37 ° for 30 min. in reticulocyte swelling buffer containing 3.0 μg./ml. of pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N.J.)

Alkaline hydrolysis was carried out in 0.3 M-KOH at 37 ° for 1 hr.

RESULTS

Effect of actinomycin D on virus production

Suspension cultures of BHK 21 cells were infected with rubella virus at an input multiplicity of 1 to 10 p.f.u. per cell. Actinomycin D (0.3 μg./ml.) was added either at the time of infection or 15 to 19 hr after infection. Samples of extracellular medium were taken at various intervals. When actinomycin D was added at the time of infection it reduced the virus infectivity threefold. However, when actinomycin D was added 15 hr after infection or later the virus infectivity did not differ from that of the controls. These results agree with those of Woods & Robbins (1968).

Rubella virus-specific RNAs in BHK 21 cells

Two virus-specific RNAs were seen consistently in sedimentation analyses of extracts in sucrose gradients. One sedimented faster than the larger ribosomal RNA (28 s) of BHK 21 cells, and was converted to acid-soluble material by ribonuclease treatment. The other virus-specific RNA was polydisperse and found at 16 to 26 s
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(Fig. 1). After ribonuclease treatment these fractions retained acid-precipitable material sedimenting at 16 to 25 S. Alkaline hydrolysis for 1 hr at 37° in 0.3 M-KOH destroyed almost all acid-precipitable material.

An approximate sedimentation coefficient for the larger specific RNA of rubella virus was obtained by infecting BHK21 cells with rubella virus as described in Methods. At 20 hr the medium was changed to phosphate-free BHK21-medium supplemented with 10% tryptose phosphate broth, 5% newborn calf serum and 0.3 μg/ml actinomycin D. At 21 hr 10 μC/ml [32P]-orthophosphate (Institutt for Atomenergi, Kjeller, Norway) was added and at 23 hr RNA was extracted. One ml.

![Figure 1](image)

**Fig. 1.** Sedimentation analysis of sodium dodecyl sulphate extract from labelled BHK21 cells infected with rubella virus. Fractions of 1 ml. were analysed for extinction at 260 nm. (E260 • • •), acid-insoluble radioactivity before (○——○) and after ribonuclease treatment (□——□) as described in Methods.

of this extract and 1 ml. of extract from [3H]-uridine-labelled BHK21 cells infected with Semliki Forest virus (Kääriäinen & Gomatos, 1969) were layered on a 15% to 30% (w/w) sucrose gradient and centrifuged for 15 hr at 52,200 g at 20°. Fractions of 1 ml. were collected and tested for 32P- and 3H-activity (Fig. 2). The larger rubella virus-specific RNA sedimented less rapidly than the 42 S RNA induced by Semliki Forest virus and thus has a sedimentation coefficient of about 38 to 40 S.

The 38 to 40 S RNA was detected as early as 15 to 17 hr after infection. At 21 to 23 hr after infection there was much more labelled material in this fraction. The RNA preparations failed to infect BHK21/WT-2 cells when tested as described by Pagano, McCutchan & Vaheri (1967).
RNA extracted from labelled rubella virus particles

In sucrose gradients the peak of haemagglutination of rubella virus, as purified from the extracellular medium of cell cultures labelled with $[^3H]$-uridine, coincided with the main peak of tritium. These peak fractions were dialysed overnight against phosphate-buffered saline and extracted with sodium dodecyl sulphate. One ml. of this extract and 1 ml. of extract from uninfected, unlabelled BHK21 cells were mixed and analysed in the ultracentrifuge as described in Methods (Fig. 3). The sedimentation velocity of the RNA extracted from virus particles was identical to that of the larger RNA extracted from labelled BHK21 cells infected with rubella virus. Additional phenol extraction after treatment with sodium dodecyl sulphate made the sedimentation pattern more heterogenous but with most of the label at 30 to 40 s.

![Fig. 2. Comparison of extract from $^{32}$P-labelled BHK21 cells infected with rubella virus with that from $[^3H]$-uridine labelled cells infected with Semliki Forest virus. Each fraction was analysed for $E_{260}$. (○ — ○), acid-insoluble $^{32}$P activity (■ — ■) and $^3$H activity (□ — □).](image)

RNA synthesis in actinomycin D-treated control cells

Labelled acid-insoluble material between 10 and 16 s was regularly found in both virus-infected and uninfected cells. This material of unknown origin was mainly sensitive to ribonuclease. Increasing the actinomycin D concentration from 0.3 to 2 μg./ml. did not alter significantly the amount of acid-insoluble material in these fractions. Tests to detect infections by bacteria, fungi or mycoplasmas (thioglycollate, Sabouraud and Hayflick’s agar tests) were negative.

**DISCUSSION**

The incorporation of $[^3H]$-uridine into rubella virus particles is direct evidence that the nucleic acid is RNA. During the preparation of this paper similar results were reported by Brodersen & Thomssen (1969). The near identity of sedimentation
properties suggests that the virus-induced 38 to 40 s RNA in cells is identical with the viral-RNA.

Replicative RNA complexes may account for some of the partially ribonuclease resistant RNA sedimenting broadly with and between the host cell ribosomal RNAs. Before and after ribonuclease treatment a component of 18 to 20 s was regularly obtained. Characterization of the postulated replicative intermediate of 16 to 26 s requires more information on the effect of ionic strength and other physicochemical conditions on the sedimentation pattern of this RNA.

![Graph](image)

**Fig. 3.** Comparison of sedimentation analyses of rubella virus-specific RNA extracted from BHK21 cells (upper part) and of RNA extracted from labelled rubella virus particles (lower part). Each fraction was analysed for E260. (● - - - ●) and acid-insoluble radioactivity (○ — — ○).

During this work W. D. Sedwick (personal communication), Wistar Institute, Philadelphia, Pa., independently detected in BHK21 cells infected with rubella virus two types of virus-specific RNA sedimenting similarly to those described in this paper.

A relationship between rubella virus and group A arthropod-borne viruses was suggested by similarities in morphogenesis and in ultrastructure of virus particles (Holmes & Warburton, 1967; von Bonsdorff & Vaheri, 1969). This is supported by
information on the RNAs of these viruses. The sedimentation coefficients of the 
RNAs of, for instance, Western Equine Encephalomyelitis virus (Sreevalsan & 
Lockart, 1966) and Semliki Forest virus (Friedman, Levy & Carter, 1966; Sonnabend, 
Martin & Mecs, 1967) are reported to be 40 to 42 s, which is close to the 38 to 40 s 
for rubella virus RNA reported here. However, instead of the 0.1 M-NaCl used for the 
arbovirus RNAs the sucrose gradient for rubella virus RNAs was made in buffer 
containing 0.05 M-NaCl and 0.0015 M-MgCl₂, which may have influenced the sedi-
mentation velocities. Semliki Forest virus (Friedman et al. 1966, Sonnabend et al. 
1967) and rubella virus induce in infected cells the synthesis of partially ribonuclease-
resistant RNAs of similar sedimentation coefficients (about 20 s). In addition, a 
ribonuclease-sensitive RNA of 26 s has been identified for both of the above arbo-
viruses but only in some experiments for rubella virus. The 26 s RNA induced by 
Western Equine Encephalomyelitis virus may, as for rubella virus, be a breakdown 
product of the 40 s RNA (Sreevalsan et al. 1968). In contrast to its many similarities 
with the RNAs of some arboviruses, the RNA of rubella virus, and the RNAs in 
cells infected with rubella virus are distinct from those of paramyxoviruses (Blair & 
Robinson, 1968).

RNA synthesis in a variety of uninfected cells in the presence of relatively high con-
centrations of actinomycin D has been reported by several workers. Martin & Brown 
(1967a, b) found that RNA of 12 to 20 s was synthesized in BHK21 cells in the 
presence of 1 µg/ml. of actinomycin D. This synthesis was greatest during the expo-
nential phase of cell growth. In cells infected with foot-and-mouth-disease virus the 
synthesis was greater than in uninfected cells. It is thus probable, especially because 
suspension cultures were maintained on a rich medium, that some RNA in fractions 
containing the partially ribonuclease-resistant rubella virus-specific RNA was not 
induced by rubella virus. The synthesis of actinomycin D-resistant RNA in BHK21 
cells may be due to the latent hamster virus (Thomas et al. 1968) known to contaminate 
these cells and frequently detected in our system. Further investigations, including 
attempted hybridization of this RNA with host DNA, are in progress.

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