NOTES

Synthesis of Viral-Specific Ribonucleic Acid in Rubella Virus-Infected Cells

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Monolayers of BHK-21/W1-2 cells were pulsed with ³H-uridine at different times after infection with rubella virus, and viral-specific cytoplasmic ribonucleic acid species were demonstrated.

Recent electronmicrographic observations of rubella virus (RV) show that the virion is spherical with an outer envelope and that it appears to mature by budding from cell membranes (5, 10). However, little is known of RV structure or mode of replication at the molecular level. We report here the synthesis of rubella virus-specific ribonucleic acid (RNA) components in BHK-21/W1-2 tissue cultures, using methods that have been developed with other RNA viruses (1, 2, 5, 8).

The preparation of RV (Gilchrist strain) pools from spinner culture and of BHK-21 cells grown in monolayer has been reported previously (12, 13). Virus plaque assays were performed by the method of Rhim et al. (11). Confluent monolayer cultures grown in 100-mm petri dishes were infected with RV at input multiplicities of 5 to 10 plaque-forming units (PFU)/cell. The production of infectious virus in cultures to which was added actinomycin D (AD) (1.0 µg/ml) at 36 hr after infection was similar to that in control cultures (Fig. 1).

To determine the time when the maximal rate of viral-specific RNA synthesis occurs, ³H-uridine incorporation into RNA in the presence of AD was measured at various times after virus infection. The rate of ³H-uridine incorporation into the AD-resistant RNA of infected cells increased with time after infection and reached a maximum at about 56 hr after infection (Fig. 2). Employing ribonuclease in 0.2 M NaCl in which single-stranded RNA is rendered acid soluble and double-stranded RNA remains acid precipitable (4), about 50% of the RNA labeled with ³H-uridine during each time period was ribonuclease susceptible, whereas the other half was resistant to

ribonuclease digestion. The time after infection at which RNA synthesis in the presence of AD is near or at the maximal rate corresponds to the

FIG. 1. Effect of actinomycin D on the growth of rubella virus in BHK-21/W1-2 cells. Replicate cultures containing about 10^6 BHK-21/W1-2 cells in 100-mm plastic dishes were infected with rubella virus. After 3 hr of virus adsorption, the cells were washed with phosphate-deficient buffer, and maintenance medium containing 10% Agamma calf serum and antibiotics were added to each culture. After incubation at 34 C for 36 hr, the medium of one culture was replaced with 10 ml of fresh medium (○) and the medium of the other was replaced with medium containing 1.0 µg/ml of AD (●). A 1 µg/ml amount of AD used in these experiments has been shown to inhibit 92% of RNA synthesis in uninfected cells within 30 min, as determined by ³H-uridine incorporation into cell RNA. At varying intervals after infection, samples were removed and titered for PFU.

time when the virus growth was maximal as measured by release of infectious virus into the culture medium (Fig. 2). At this time, microscopic examination of the cultures reveals about 2+

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FIG. 2. Incorporation of ^3H -uridine into RNA of BHK-21/W1-2 cultures in the presence of actinomycin D at different times after infection with rubella virus. Five parallel BHK-21 cultures were infected with rubella virus as described in the legend of Fig. 1. At different times after infection, the medium of different cultures was replaced with 5 ml of medium containing 20 μg of AD per ml. This amount of AD was used for these experiments to inhibit ^3H -uridine incorporation into uninfected cells by more than 99%. At indicated time points, each culture was incubated with AD for 30 min, and then 100 μc of $5'\text{-}^3\text{H}$ -uridine (25 c/mmole) in a volume of 5 ml was added. After 60 min more of incubation, monolayers were washed three times with phosphate-deficient buffer and the cells were removed from the plates with 4 ml of buffer containing 0.1 M sodium acetate (pH 5.2) and 0.5% sodium dodecyl sulfate. Each sample of cell material was then extracted three times with an equal volume of standard buffer-saturated phenol [0.1 M NaCl, 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.0, 0.005 M ethylenediaminetetraacetate (EDTA), and 0.02% 2-mercaptoethanol (v/v)], and the nucleic acid remaining in the aqueous phase was precipitated twice with 2 to 3 volumes of cold ethyl alcohol. The nucleic acid was redissolved in a small volume of standard buffer (without 2-mercaptoethanol), and a sample was removed for determination of radioactivity (total counts/min) by trichloroacetic acid precipitation of the nucleic acid, collection and washing of the precipitate on a membrane filter, and counting in toluene scintillation fluid (2). For ribonuclease treatment, a sample of not more than 50 μg of RNA per ml of buffer (0.2 M NaCl, 0.01 M Tris-hydrochloride, pH 7.0, and 0.005 M EDTA) was incubated with 10 μg of bovine pancreatic ribonuclease for 30 min at 35 C before determining trichloroacetic acid-precipitable radioactivity. The amount of trichloroacetic acid-soluble RNA counts

FIG. 3. Sucrose density gradient sedimentation of the RNA from rubella virus-infected (Fig. 3b) and uninfected (Fig. 3a) BHK-21/W1-2 cells. The media of an uninfected culture and of a parallel culture 50 hr after infection with rubella virus (infected as described in Fig. 1) were each replaced with 5.0 ml of medium containing 20 μg of AD per ml. After incubation for 30 min, 500 μc of $5'\text{-}^3\text{H}$ -uridine (25 c/mmole) in a volume of 5.0 ml was added to each culture. After 60 min more of incubation, the nucleic acid from each culture was prepared by phenol extraction and alcohol precipitation as described in Fig. 2. The precipitates were redissolved in 0.4 ml of buffer, and 0.2 ml of each was layered over a 13.5-ml, linear, 5 to 20% sucrose gradient (1). The two gradients were then centrifuged at 40,000 rev/min for 5 hr at 3 C in a Spinco SW40 Ti rotor, and then 40 equal fractions were collected from the bottom of each tube. The ultraviolet absorbance at 260 nm of the fractions from both gradients was determined. Each fraction of the gradient containing RNA from the RV-infected culture was divided in half for determination of trichloroacetic acid-precipitable radioactivity before and after ribonuclease digestion, as described in Fig. 2. Each whole fraction from the gradient containing RNA from the uninfected culture was sampled to determine trichloroacetic acid-precipitable radioactivity. The sedimentation was from right to left in these figures.

was determined as the difference between trichloroacetic acid-precipitable RNA after ribonuclease treatment and total radioactivity counts without ribonuclease treatment. The cytopathogenic effects, or degree of granularity and percentage of cells attached to the dish, were observed microscopically and are designated 0, +, 2+, 3+, or 4+ when they involved 0, 25, 50, 75, or 100% of the cells, respectively.

cytopathogenic effect, and 80% of the cells remain attached to the culture plate. Thus, in RV-infected cells new viral specific RNA synthesis can be detected which is not inhibited by AD and which increases in rate in parallel with the virus growth curve. AD-resistant RNA with the properties of both single- and double-stranded RNA is synthesized.

When further studied by sucrose density gradient sedimentation (Fig. 3b) four RV-specific RNA components, designated I to IV, are observed in infected cells, which are estimated to be about 41, 29, and 24S for single-stranded species and about 19S for double-stranded RNA species, respectively. The *S* values were calculated by the method of Martin and Ames (8). It can be seen that almost all of the radioactive RNA in the 41S component is rendered trichloroacetic acid-soluble by ribonuclease under these conditions, and around 70 to 80% of the ³H-RNA in the 19S region remains trichloroacetic acid-precipitable after ribonuclease digestion. A smaller fraction of the ³H-RNA in the 29 and 24S regions is resistant to ribonuclease digestion. The ³H-uridine was not incorporated into these distinct RNA components in uninfected cells (Fig. 3a), although a small amount of labeled RNA sedimenting heterogeneously between 4 and 28S is present in uninfected cells under the conditions used.

The data in Fig. 1 indicate that even when BHK-21 cells were infected at multiplicity of 5 to 10 PFU/cell, no more than 5 PFU/cell of virus were produced in the first 36 to 48 hr after infection. The low yield is due in part to the small number (about 3%) of the BHK-21 cells initially infected under our conditions, as shown by an infectious center assay. Similarly, low yields have been obtained with other cell types such as VERO cells, although higher virus titers have been reported by using roller bottle methods (7). The low rate of virus production is also reflected by the low rate of viral-specific RNA synthesis in RV-infected cells, and this has limited the experi-

ments that we have been able to do with RV replication in tissue culture. On the other hand, we have been able to detect the newly synthesized viral-specific RNA species in BHK-21/W1-2 cells as described in this note and in VERO cells (*unpublished data*).

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