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Isolation of RNA Species from HeLa Cells Infected with Respiratory Syncytial Virus (38089)

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Respiratory syncytial virus (RSV), the major viral respiratory pathogen of infancy, was isolated and first recovered from patients with pneumonia in 1956 (1, 2). Subsequent studies have indicated that it is an enveloped, RNA-containing virus which, like many paramyxoviruses, can produce syncytia in tissue culture cell monolayers (2, 3). When examined by electron microscopy, RSV resembles viruses of the paramyxovirus group in its overall size, in the spikelike projections from its envelope, and in the helical configuration of its nucleocapsid (4). The diameter of the RSV nucleocapsid and the pitch of its nucleocapsid helix, however, differs from those of the paramyxoviruses (4–6). It appears that the nucleocapsid of RSV resembles most closely that of pneumonia virus of mice (7). RSV also differs from the paramyxoviruses in that it lacks a demonstrable hemagglutinin or neuraminidase (8). The taxonomic status of RSV is further clouded because the difficulty in obtaining high-titered suspensions of purified virus has hindered biochemical characterization.

Recently, a number of investigators have shown that infection of cultured cells with viruses of the paramyxovirus group results in the actinomycin D-resistant synthesis of RNAs which sediment more slowly than virion RNA (9–12). We report here on attempts to study RNA synthesis in RSV-infected HeLa cells which have been treated with actinomycin D.

Methods. Three day-old confluent monolayers of HeLa cells grown in 32-oz bottles (Flow Laboratories, Rockville, MD) were inoculated with 1 ml of a suspension of RSV A2 strain (passaged 5 times in human embryonic kidney culture, 4 times in Hep-2 cells, and once in HeLa cells) containing approximately 2×10^7 plaque-forming units (PFU)/ml. This gave a multiplicity of input (m.o.i.) of approximately 1 PFU/cell. After 90 min of adsorption at 20°C, the cultures were overlaid with 30 ml of Eagle's medium number 2 containing 2% inactivated agamma calf serum, glutamine (2 m*M*), penicillin (250 U/ml), streptomycin (250 mg/ml), and amphotericin B (50 U/ml). Cultures were then incubated at 37°C.

At the indicated time intervals, actinomycin D was added to cultures to achieve a concentration of 5 μg/ml and the cultures were returned to the incubator for 90 min. Preliminary experiments had indicated that such treatment depressed host-cell RNA synthesis by 98.6%. ^{3}H-uridine (Schwartz-Mann, 27 Ci/mM) was then added to give a final concentration of 15 μCi/ml and labeling was allowed to proceed for 2 hr at 37°C. At the end of this period of time the cultures were washed twice with phosphate-buffered saline (PBS) and the cells scraped into fresh PBS and centrifuged at 2000 rpm for 10 min. RNA was extracted from the resulting pellet according to the SDS–hot phenol method of Scherrer (13). RNA extracted in this manner gave OD_{260}/OD_{280} ratios of approximately 2:1.

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For sedimentation analyses, the precipitated RNAs were resolubilized in a buffer containing 0.01 *M* EDTA, 0.05 *M* NaCl, and 5 μg/ml of polyvinyl sulfate (PVS). One-tenth of a milliliter of this solution was then layered onto a preformed gradient of 5–20% sucrose in 0.01 *M* EDTA–0.05 *M* NaCl and centrifugation was carried out at 40,000 rpm for 4 hr using a Spinco SW40 rotor in a Spinco L2-65B preparative ultracentrifuge. Twenty-five-drop (0.5-ml) fractions were then collected by puncturing the bottom of the tube and RNA was precipitated with 2.3 ml of cold 7% trichloroacetic acid after the addition of 200 μg of a carrier yeast RNA (Schwartz-Mann). The precipitates were then collected on Millipore filters, washed with ethanol, dried, and radioactivity counted in a Packard model 527 liquid scintillation counter. Sedimentation coefficients were estimated by comparing the sedimentation pattern of the experimental material with the pattern produced by HeLa cell RNAs (28S, 18S, 4S) as described by Scherrer and Darnell (14).

Results. Analysis of RNAs in uninfected HeLa cells, not exposed to actinomycin D, revealed the pattern of 28S, 18S, and 4S species previously described (14). In contrast, cells infected 24 hr previously with RSV contained reduced amounts of RNAs with 4S species being most abundant (Fig. 1E). This finding suggests that RSV infection depresses cellular RNA synthesis. We can only speculate about the mechanism by means of which the virus accomplishes this depression of cellular RNA synthesis. Perhaps it is an early manifestation of the process which eventually brings about cell death in RSV infection.

In experiments employing actinomycin D, it was found that the actinomycin D-resistant incorporation of ^{3}H-uridine into acid-precipitable material in RSV-infected cells was low. However, definite reproducible peaks of activity could be detected in the sucrose gradients and the contrast with the results obtained in uninfected cells was striking. Twelve hours after inoculation, cultures infected with RSV showed little more incorporation of ^{3}H-uridine than did uninfected cultures (Fig. 1A). By 18 hours, infected cultures showed gradually increasing synthesis of material which banded broadly on gradient analysis (Fig. 1B). At 24 hr, a clear peak at approximately 10S was present, and by 36 hr, when incorporation was maximal, a second peak was present, at approximately 28S (Fig. 1C and D). Cultures incubated for a longer period of time did not incorporate significant amounts of ^{3}H-uridine as the infected monolayers were almost completely destroyed. Heavier (c. 50S) RNA species, characteristic of paramyxovirus virion RNAs, were never detected. The nucleic acid extracted from the infected cells at 36 hr was subjected to alkaline hydrolysis by incubating 0.25 ml of the resolubilized ethanol precipitate with 0.25 ml of 0.6 *M* NaOH for 24 hr at 37°C. This treatment reduced the acid-precipitable counts by 98.8%, indicating that the bulk of the labeled nucleic acid was RNA rather than DNA.

In order to determine the strandedness of the RNA, the material extracted at 36 hr was redissolved in 0.01 *M* Tris buffer and 0.01 *M* NaCl (pH 7.3), dialyzed overnight against this buffer to remove traces of PVS and EDTA, and 0.05 ml of the dialysate incubated at 37°C for 2 hr with 1.3 U of *N. Crassa* nuclease (kindly provided by Dr. George Fareed, Department of Biological Chemistry, Harvard Medical School, Boston, MA) in the presence of 0.01 *M* $MgCl_2$. This treatment reduced the acid-precipitable radioactivity by 81%, indicating that the bulk of the RNA was single stranded. Because of the limited number of counts in the original material, the sedimentation characteristics of the *N. Crassa* nuclease-resistant species could not be determined.

Discussion. Slowly sedimenting RNAs have been detected in cells infected with a number of paramyxoviruses, viz., Newcastle disease virus (9), Sendai (10), mumps (11), and measles (12). The principle species found have been 18S and 28–35S. The production of c. 10S and 28S has been noted soon after the infection of cell monolayers with measles virus, with 18S and 33S species appearing later (12). The

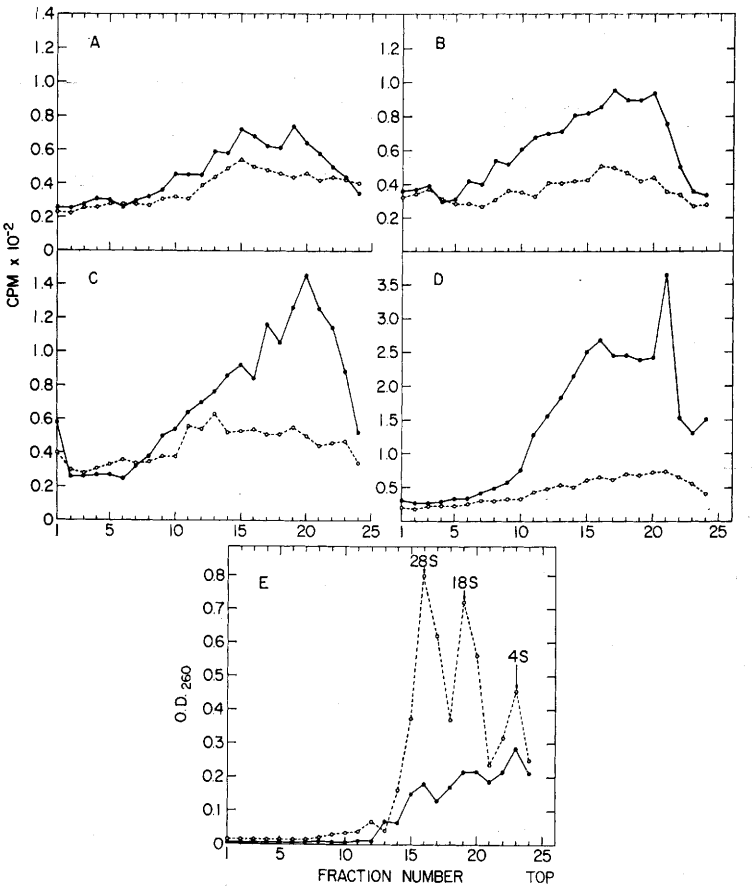

FIG. 1. Three day-old monolayers of HeLa cells were infected with the A2 strain of RSV. At the indicated times, the infected cultures (—●—) and corresponding control cultures (—○—) were pretreated for 90 min with 5 μg/ml of actinomycin D and then labeled for 2 hr with ^{3}H-uridine (15 μCi/ml). RNA was then extracted according to the method of Scherrer (13) and analyzed on preformed gradients of 5–20% sucrose. Fractions were collected by puncturing the bottom of the tubes and acid-precipitable radioactivity was determined. Patterns of RNA incorporation at 12, 18, 24, and 36 hr postinfection respectively (A–D). RNA extracted in the absence of actinomycin D at 24 hr analyzed on a parallel gradient with respect to OD_{260} (E).

slowly sedimenting species which have been examined have been determined to be predominantly single stranded (9, 11, 15). Some partially base-paired RNAs, possible replicative and transcriptive intermediates, have also been described (11, 16, 17). The slowly sedimenting species have been determined to be complementary to virion RNA in those instances in which such material was available for annealing studies (9–11). Paramyxovirus virion RNAs are characteristically in the 50S range (18). Such RNAs have seldom been noted to become the predominant species in paramyxovirus-infected cells (9–12, 19) and, with certain strains, under certain conditions, and at certain stages of infection, 50S RNA has not been detected (12, 20, 21).

The RNAs produced in RSV-infected cells resemble those described for cells infected with established members of the paramyxovirus group. In RSV-infected HeLa cells, ^{3}H-uridine is incorporated into slowly sedimenting, acid-precipitable species which

sediment principally at c. 10S and 28S. This incorporation is maximal and the RNA species are most clearly defined at 36 hr after infection. Such incorporation does not occur in uninfected cells. Analysis by alkaline hydrolysis and by *N. Crassa* nuclease digestion indicates that the species produced in RSV-infected cells are principally single-stranded RNAs. Sedimentation analysis of the nuclease-resistant species has been hampered by lack of a sufficient amount of this material.

It is possible that more rapidly sedimenting species appear later in the course of RSV infection as has been noted late in the course of measles infection (12). Unfortunately, the rapid destruction of RSV-infected cells after 36 hr has prevented a search for this late type of RNA.

Since purified RSV has not been obtained in high enough concentration to allow for the extraction of virion RNA, it has not been possible to perform the relevant hybridization experiments to ascertain whether or not the species obtained from the infected cells are in fact RSV-coded. We did not note ^{3}H-uridine incorporation into any species sedimenting in the 50S region.

More definitive comparisons of RSV biochemistry with that of the known paramyxoviruses must await the production of more concentrated virus suspensions or other technical advances.

Summary. HeLa cells infected with RSV at an m.o.i. of 1 PFU/cell were incubated for 36 hr and then labeled with ^{3}H-uridine for 2 hr in the presence of actinomycin D. RNAs sedimenting at c. 10S and 28S could be extracted from the infected cells, whereas there was no significant incorporation of ^{3}H-uridine in uninfected cells treated in a similar fashion. The RNA obtained from the RSV-infected cells was apparently about 80% single stranded. These findings resemble those described for members of the paramyxovirus group.

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