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Nucleic Acids of Respiratory Syncytial Virus

DENNIS M. LAMBERT,* MARCEL W. PONS, GUSTAVE N. MBUY, AND KAROLINE DORSCH-HASLER†

Department of Molecular Virology, The Christ Hospital Institute of Medical Research, Cincinnati, Ohio 45219

Analysis of purified respiratory syncytial virus revealed that the virion RNA was composed of 50S, 28S, 18S, and 4S species. The 18S and 28S species were presumed to represent host rRNA since virus grown in actinomycin D-treated cells contained only 50S and 4S RNAs. Actinomycin D treatment stimulated production of infectious respiratory syncytial virus 5- to 10-fold. The 50S virion RNA was shown to hybridize with polyadenylated mRNA's isolated from infected cells, indicating that respiratory syncytial virus RNA is of negative-strand sense. Six mRNA's were identified by polyacrylamide gel electrophoresis.

Respiratory syncytial (RS) virus isolation from humans was first reported in 1957 by Chanock et al. (7). Since that time, RS virus has been shown to be the principal etiological agent associated with respiratory illness in the very young (6). However, relatively little is known about the physicochemical properties of the virion or the biochemistry of its replication. The virion resembles the paramyxoviruses in gross physical appearance as demonstrated by electron microscopy (1, 3, 15), but it differs from this group of viruses in several important aspects. RS virus lacks a demonstrable hemagglutinating or neuraminidase activity (6, 22), and its nucleocapsid differs from that of the myxoviruses and paramyxoviruses in diameter and in helical pitch (10, 26). The proper classification of this virus is still uncertain. Melnick (19) suggested placing RS virus in the metamyxovirus group separate from the ortho- and paramyxoviruses. The International Committee on Taxonomy of Viruses recently recommended that RS virus be placed in the genus *Pneumovirus* (which also includes bovine RS virus and pneumonia virus of mice) within the family Paramyxoviridae (16).

Numerous studies have been conducted to determine the size of the RS genome (24, 27; E. J. Hoffman, E. C. Ford, and J. L. Gerin, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, S300, p. 262). None of these reports have been conclusive because in almost all cases numerous size classes of RNA were resolved, and the approximately 50S RNA was either minor compared with the other RNA components or was not detected. Wunner et al. (24) observed that RNA from virus-infected cells, labeled in the presence of 2.5 μg of actinomycin D per ml, contained a main component of 16S and a minor 50S component. Under somewhat similar conditions, Hodes et al. (14) reported that the main species of RNA found in infected cells was a 10S RNA with 28S species appearing only later in infection. Wunner et al. (24) also reported the presence of a minor fast-sedimenting RNA component (50S) in virions. An RNA component sedimenting at 52S was isolated from both RS virions and ribonucleoprotein by Zhdanov et al. (27). These data suggested, but did not conclusively demonstrate, that the genome of RS virus is similar in size to that of other paramyxoviruses.

Biochemical studies of RS virus have been hampered because the virus is extremely fragile and therefore very difficult to purify and characterize. Because of this, a rigorous investigation of the nature of the viral genome has not been previously carried out. Previous observations that actinomycin D treatment of infected cells stimulated RS virus release (8, 23) were confirmed in this study for the HEp-2 cell system. Actinomycin D treatment of infected cells also allowed a means for investigating the genomic RNA of virions while inhibiting incorporation of label into most host RNA species. In addition, we have developed a purification scheme for RS virus which allows retention of infectivity and yields RS virions of fairly high purity. In this report RS virion RNA and virus-specific mRNA from infected HEp-2 cells have been characterized.

MATERIALS AND METHODS

Cells and virus. HEp-2 cells susceptible to RS virus were obtained from Flow Laboratories, Inc., Rockville, Md. The cells were grown as monolayers in stationary culture in Eagle minimal essential medium (MEM) containing nonessential amino acids (Flow Laboratories) and 10% fetal calf serum at 36°C. The

† Present address: Department of Microbiology, Columbia University College of Physicians and Surgeons, New York, NY 10027.

Long strain of RS virus, generously supplied by R. Chanock (National Institutes of Health, Bethesda, Md.), was used throughout this study.

For infection, HEp-2 cells, either in flasks (150 cm^2) or in roller bottles (1,100 cm^2), were inoculated with RS virus diluted in Earle balanced salt solution containing 1% fetal bovine serum at an input multiplicity of infection of 0.02 to 0.2 PFU per cell. After 60 to 90 min at 36°C the inoculum was removed, MEM containing 3% fetal calf serum was added, and the cells were incubated at 36°C. At 4 h postinfection (p.i.) 0.5 μg of actinomycin D (Sigma) was added for 1 h at 36°C, after which the medium was replaced with drug-free MEM containing 3% fetal bovine serum. L-^{3}H-amino acids (10 μCi/ml of MEM containing 10% normal complement of L-amino acids) or [5-^{3}H]uridine (10 μCi/ml) was added at this time for the production of labeled virus. Under these conditions, syncytia were usually visible after 24 h although the infection was usually allowed to proceed for 48 to 72 h when syncytium formation was complete.

The titer of extracellular virus was usually 1×10^6 to 5×10^6 PFU/ml. Unlabeled high-titer virus stocks were prepared by replacing the medium at 48 h with 10 ml of serum-free Earle saline per infected roller bottle and incubating overnight at 36°C. The virus was harvested and clarified at 2,000 rpm for 10 min in a J-6 centrifuge (Beckman Instruments, Inc., Fullerton, Calif.) and then adjusted to 10% fetal bovine serum and quick-frozen in a dry ice-ethanol bath. Virus stocks were stored at −70°C. Titers of virus prepared in this way were, after freezing, usually 2×10^7 to 5×10^7 PFU/ml. When virus was to be purified, the Earle saline from the overnight incubation was pooled with the previously harvested culture fluids and cells, and debris was removed by centrifugation at 2,000 rpm for 10 min.

Virus purification. Unlabeled virus in the supernatant from the low-speed centrifugation was concentrated 10-fold in an Amicon DC-2 concentrator. Virus remaining in the concentrator fibers was removed by rinsing with STEU buffer (0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-hydrochloride [pH 7.2], 1 M urea). The rinse material (usually 50 ml) was pooled with concentrated virus. Concentrated unlabeled virus as well as virus labeled with [5-^{3}H]uridine or L-^{3}H-amino acids were layered over discontinuous gradients composed of 1 ml of 60% and 8 ml of 30% sucrose in STEU buffer and centrifuged at 25,000 rpm for 1 h in an SW27 rotor (Beckman) at 5°C. Virus at the 30 to 60% sucrose interfaces was collected from the top of the tubes after removing the supernatant material. Unless otherwise specified, the virus was diluted to 6 ml with STEU, homogenized by 20 strokes with a motor-driven Teflon Dounce homogenizer, and then sonicated in 50-ml plastic centrifuge tubes at 50% power for 15 s in a cup probe (VirSonic) filled with water. Samples were centrifuged at 5,000 rpm for 10 min in a JA-20 rotor. Supernatants were layered over discontinuous gradients composed of 2 ml of 60% and 10 ml of 30% sucrose in STEU buffer and centrifuged at 25,000 rpm for 1 h. The virus at the 30 to 60% interface was collected by puncturing the bottom of the tubes and collecting the visible bands. Virus was diluted in STEU buffer and layered through 5 ml of mineral oil onto 20-ml linear gradients of 20 to 60% sucrose in STEU buffer. The gradients were centrifuged in an SW27 rotor at 22,000 rpm for 17 h or at 25,000 rpm for 0.5 h and 1-ml fractions were collected from the bottom of the tubes. Radioactivity of labeled virus samples was measured by counting 50-μl samples in 5 ml of Bray scintillation fluid.

Peak fractions were pooled, diluted to 34 ml with STEU buffer, and concentrated onto a 1-ml 60% sucrose cushion in STE (0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-hydrochloride [pH 7.2]). The virus was further analyzed on gradients, or the RNA was extracted as described below.

Infectivity assays. Virus infectivity was determined by plaque assay in confluent HEp-2 cell monolayers in 60-mm plates overlaid with 5 ml of 0.5% (wt/vol) agarose-MEM overlay containing 3% fetal bovine serum. After 4 days of incubation at 36°C in a 5% CO_2 atmosphere, plates were again overlaid with 5 ml of agarose-MEM containing neutral red (1:15,000) and incubated at 36°C. Plaques were counted 4 to 12 h later as darkly stained syncytia. An alternative plaque assay method utilized 0.5% (wt/vol) methylcellulose-MEM overlay containing 3% fetal bovine serum. The plates were incubated for 48 h at 36°C in a CO_2 incubator, at which time the cells were fixed with 10% Formalin for 30 min and stained with 50% hematoxylin for 1 h followed by 2% eosin for 30 min. Plaques were evident as pink syncytia in a purple-stained background of normal cells.

Extraction of viral nucleic acid. After determining the location of virus in linear sucrose gradients, the radioactive peak fractions were pooled and treated with 500 μg of proteinase K (EM Biochemicals) per ml for 20 min at 37°C. After adding 0.1 volume of 10× SLA buffer (5% sodium dodecyl sulfate, 1.4 M $LiCl_2$, 5×10^{-2} M sodium acetate [pH 4.9]) and incubating at 37°C for 10 min, deproteinization was accomplished by adding an equal volume of redistilled phenol saturated with STE, shaking for 1 min, and then adding 0.5 volume of chloroform-isoamyl alcohol (24:1) and shaking for 4 min. After centrifugation, the organic phase was extracted with an equal volume of 1× SLA buffer, the two aqueous phases were pooled, and the RNA was precipitated with 2.5 volumes of 95% ethanol. After overnight precipitation the RNA was pelleted by centrifuging at $2,000 \times g$ for 20 min, and the RNA pellet was washed once with ethanol. The final RNA precipitate was dissolved in 1 ml of STE buffer.

Sedimentation analysis of viral RNA. Nucleic acids were analyzed on linear 32-ml 10 to 35% (wt/vol) glycerol gradients in STEU by centrifugation in an SW27 rotor for 17 h at 22,000 rpm. The sedimentation markers used were [5-^{3}H]uridine-labeled HEp-2 cell 28S and 18S rRNA's. Viral RNA was further characterized by sedimentation on 5 to 20% (wt/vol) sucrose gradients containing STE (pH 4.9) by centrifugation in an SW41 rotor for 3 h at 40,000 rpm or in an SW27 rotor for 17 h at 17,000 rpm. Gradient fractions were collected from the bottoms of the tubes.

Isolation of RS RNA from infected cells. Confluent HEp-2 cell monolayers were infected with RS virus at a multiplicity of 2 PFU per cell. Identical uninfected cultures were used as controls. At 4 h p.i.

control and infected cultures were treated with 5 μg of actinomycin D per ml for 1 h at 36°C, and then 100-μCi/ml [5,6-^{3}H]uridine was added to each without removing the drug. At 23 h p.i. cells were harvested by scraping into the medium and centrifuging at 1,000 × *g* for 10 min. Cells were washed once with reticulocyte swelling buffer (RSB; 0.01 M Tris-hydrochloride [pH 7.2], 0.01 M NaCl, 1.5 mM $MgCl_2$) and then suspended in RSB and allowed to sit on ice for 10 min. Cells were homogenized with 20 strokes of a tight-fitting glass Dounce, and the cell homogenate was made 1% Nonidet P-40. After 5 min on ice, nuclei and cytoplasmic fractions were separated by centrifuging at 1,000 × *g* for 5 min. One milliliter of 5× and 5 ml of 1× extraction buffer (1× buffer is 2% sodium dodecyl sulfate, 0.02 M EDTA, 0.1 M Tris-hydrochloride [pH 9.0]) were added to the cytoplasmic fraction, and RNA was extracted with equal volumes of redistilled phenol (equilibrated with pH 9.0 buffer) and chloroform. The RNA was precipitated with 2.0 volumes of 95% ethanol overnight at −20°C. The pelleted RNA was redissolved in 1 ml of STE and analyzed on 5 to 20% sucrose gradients containing STEU buffer.

Removal of polyadenylate from mRNA by RNase H treatment and polyacrylamide gel electrophoresis of RNA. RNA obtained from infected and control cells by phenol extraction at pH 9.0 was chromatographed on 0.3-g oligodeoxythymidylate [oligo(dT)]-cellulose columns. The bound fractions containing mRNA were precipitated with ethanol and dissolved in 0.6 ml of a solution containing 0.1 M KCl, 0.01 M $MgCl_2$, 0.05 M Tris-hydrochloride (pH 8.0), and 50 μg of oligo(dT) (P-L Biochemicals, Milwaukee, Wis.). After incubation for 2 h at 37°C, 20 μl of solution containing 0.05 M Tris-hydrochloride (pH 7.5), 0.4 M NaCl, 0.03 M $MgCl_2$, 1 mM dithiothreitol, 50% glycerol, and 0.4 U of RNase H (P-L Biochemicals) was added, and the incubation at 37°C continued for 1 h. This is a modification of the procedure described by Etkind et al. (11). After digestion of the polyadenylate regions the samples were extracted with phenol at pH 4.9, precipitated with ethanol, collected by centrifugation, washed with 95% ethanol, and subjected to polyacrylamide gel electrophoresis in gels containing 6 M urea (21). Scintillation fluorography of gels was carried out as described by Bonner and Laskey (4).

RNA hybridization. Unlabeled RNA from uninfected and infected cells was extracted with buffered phenol (pH 9.0) and chloroform for optimal isolation of polyadenylated RNA (12). The polyadenylated RNA was isolated on oligo(dT)-cellulose columns (2) [usually 0.3 g of type 2 oligo(dT)-cellulose per column (Collaborative Research, Inc., Waltham, Mass.)], and after elution of the bound RNA it was precipitated with 2.0 volumes of 95% ethanol. RNA samples were dissolved in water and frozen at −20°C until ready for use. [^{3}H]uridine-labeled 50S viral RNA (2,000 cpm) was added to infected and uninfected cell RNAs; the mixture was heated at 100°C for 1 min, chilled quickly, adjusted to 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 50% formamide, and incubated for 48 h at 50°C. The extent of hybrid formation was determined by treating half of each sample with 5.0 μg of RNase A and 0.5 μg of RNase T_1 per ml at 37°C for 20 min followed by trichloroacetic acid precipitation of enzyme-treated and untreated samples.

RESULTS

Purification of RS virions. RS virus was purified from the medium of HEp-2 cell roller bottle cultures infected at low multiplicity (0.2 PFU per cell). The purification procedure eliminated contaminating host material while allowing efficient recovery of infectious virus. When freeze-thawed [^{3}H]uridine-labeled control cell material was copurified with virus, no label was found in the final linear sucrose gradient (data not shown). The recovery of purified virus was about 18% of the total extracellular infectious RS virus in the original culture fluids (Table 1). The threefold increase in virus titer after sonication (Table 1, steps IV and V) was probably due to the release of additional infectious virus contained in large membrane-bound vesicles seen by electron microscopy before but not after sonication (Fig. 1A and B). Virus from the final linear gradient contained no vesicles and appeared to be homogeneous. The vesicles are most likely the result of either extensive cell damage occurring late in the infection or the release of virus into intracellular vesicles which were released intact upon disruption of the cells late in infection. The purification procedure re-

TABLE 1. *Recovery of infectious RS virus at different steps in the purification procedure*

Purification step	Total PFU	Total protein (mg)[a]	PFU/mg of protein	Recovery of infectivity (%)
I. Culture medium	2.10×10^8	1,177.2	1.78×10^5	100.0
II. Amicon concentrate	2.09×10^8	1,031.2	2.03×10^5	99.5
III. First discontinuous gradient	2.40×10^7	27.5	8.73×10^5	11.4
IV. Second discontinuous gradient (before sonication)	1.60×10^7	2.5	6.40×10^6	7.6
V. Second discontinuous gradient (after sonication)[b]	4.80×10^7	2.0	2.40×10^7	22.8
VI. Linear 20 to 60% sucrose (peak fractions)	3.80×10^7	0.8	4.75×10^7	18.1

[a] Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories).
[b] In some experiments, virus was sonicated after the first discontinuous gradient.

sulted in an overall purification of approximately 270-fold on the basis of PFU per milligram of protein, whereas the total amount of protein in the virus sample was reduced by approximately 1,500-fold.

[5-^{3}H]uridine- or ^{3}H-amino acid-labeled virus purified as described banded at a density of 1.19 to 1.21 g/ml in 20 to 60% equilibrium sucrose gradients with no evidence of extraneous host materials present. Velocity sedimentation analyses were similar in that there was no evidence of extraneous RNAs or proteins and that radio-

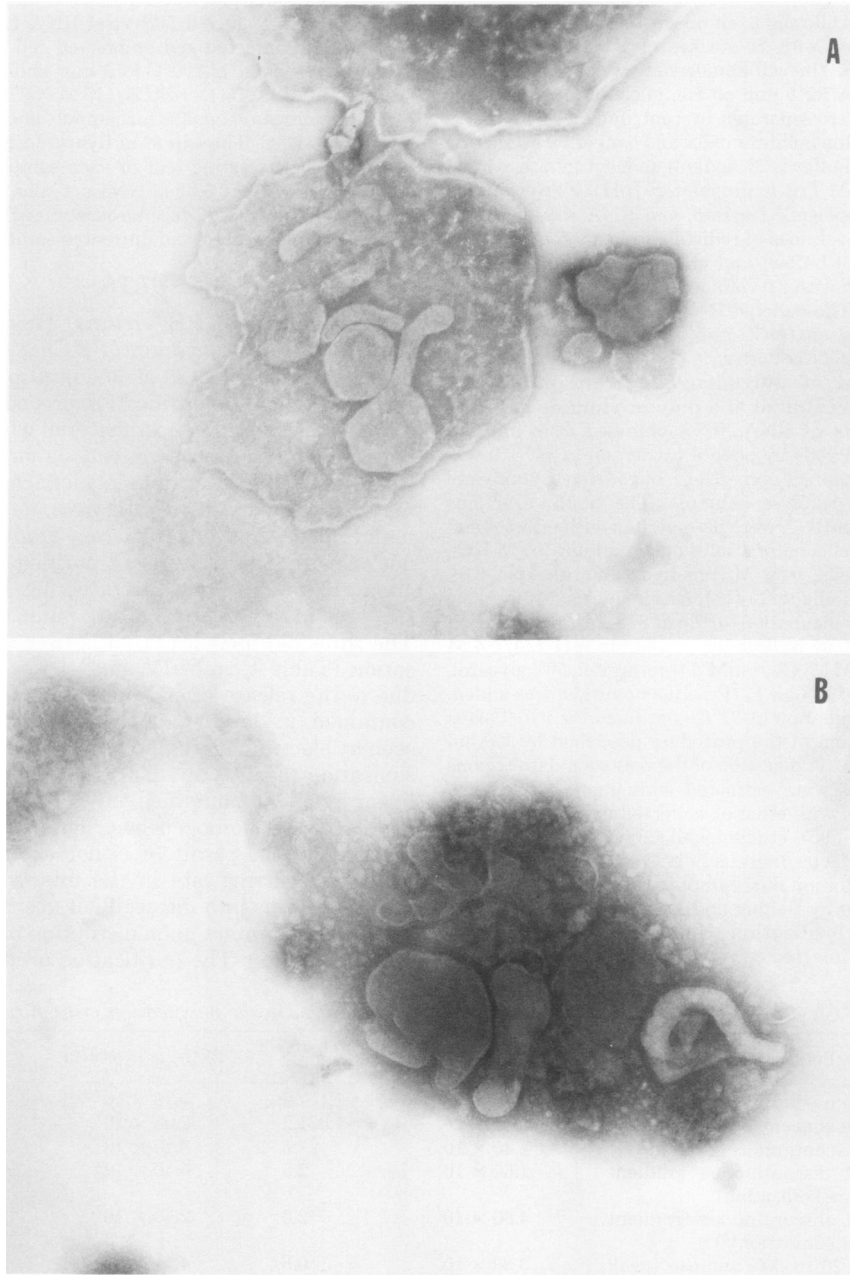

FIG. 1. *Electron microscopy of unsonicated (A) and sonicated (B) RS virus. Virus was stained with 2% phosphotungstate, placed on carbon-coated, Parlodion-treated grids, and observed in a Phillips 101 electron microscope. Final magnification: (A) ×54,000; (B) ×45,000.*

active and infectivity peaks coincided exactly.

Isolation of virion RNA. [5-^{3}H]uridine-labeled viral RNA was extracted from purified virions. As shown in Fig. 2A, proteinase K-treated viral RNA, sedimented through 10 to 35% glycerol gradients for 17 h at 22,000 rpm in an SW27 rotor, was resolved into four peaks sedimenting at 40S, 28S, 18S, and 4S. The sedimentation values were determined by cosedimentation of 18S and 28S HEp-2 cell rRNA's in identical glycerol gradients.

Since the high viscosity of glycerol in the lower portions of these gradients leads to underestimates of sedimentation values, the 40S RNA was pooled (fractions 5 to 8), precipitated with alcohol, dissolved in 1 ml of STE buffer, and sedimented through 5 to 20% sucrose gradients containing STEU buffer (Fig. 2B). As can be seen, a homogeneous peak of [5-^{3}H]uridine-labeled material sedimented at about 50S relative to 28S rRNA. This sedimentation value suggests a molecular weight of the viral RNA of approximately 5×10^6 to 6×10^6.

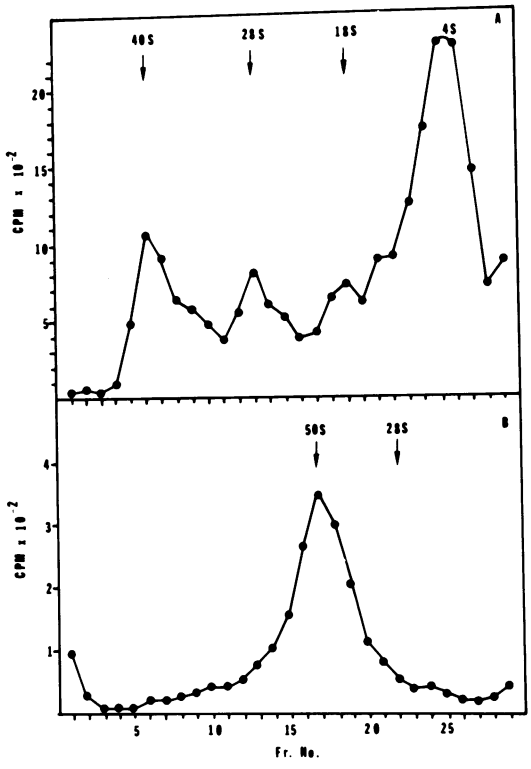

FIG. 2. *Sedimentation analysis of RS virus RNA extracted from sucrose gradient-purified virions. (A) [5-^{3}H]uridine-labeled RNA was centrifuged on 10 to 35% (wt/vol) glycerol gradients containing STEU medium for 17 h at 22,000 rpm. [5-^{3}H]uridine-labeled HEp-2 cell 28S and 18S rRNA's were used as sedimentation markers in separate gradients run at the same time. (B) The RNA from the 40S region (fractions 5 to 8) of the glycerol gradient above was pooled, ethanol precipitated, and diluted in STEU buffer. The RNA was layered over linear 5 to 20% (wt/vol) sucrose gradients containing STEU and centrifuged at 5°C in an SW41 rotor for 3 h at 40,000 rpm.*

Effects of actinomycin D treatment. Actinomycin D treatment (0.5 μg/ml) of infected cells for 1 h was found to stimulate the yield of infectious virus from HEp-2 cells. Stimulation of RS virus growth by actinomycin has been previously reported (8). We have observed as much as 19-fold stimulation (data not shown) of infectious RS virus, depending upon the time of addition of the drug, but most often a 5- to 10-fold increase in virus yield was obtained. The onset of syncytium formation was accelerated by drug treatment especially at low multiplicities of infection (0.2 PFU per cell). The growth curves of RS virus released from untreated cells and cells treated with 0.5 μg of actinomycin D per ml for 1 h are shown in Fig. 3. The release of infectious virus by actinomycin D-treated cells preceded release of virus by untreated cells by at least 2 h, and approximately fivefold more virus was released by 48 h p.i. Actinomycin D treatment was used routinely to grow virus since greater yields of infectious virus and much greater recovery of high-molecular-weight viral RNA was obtained (see below). The actinomycin D treatment (0.5 μg/ml added at 4 h p.i. for 1 h) that was selected for growth of labeled virus gave the most reproducible and optimal stimulation of infectious virus.

To determine whether the 28S and 18S RNAs found in RS RNA preparations (Fig. 2A) represented part of the virus genome or were host rRNA's, virus was grown in cells treated with actinomycin D for 1 h, after which drug-free medium containing [5-^{3}H]uridine was added. Virus was purified from both treated and untreated roller bottle culture fluids at 72 h p.i., and the RNA was extracted.

Figures 4A and B show the effects of actinomycin D treatment on the size classes of labeled RNA found in RS virions. The amount of 50S RNA recovered from virus grown in actinomycin D-treated cells was about fourfold greater than that recovered from virus grown in untreated cells. The RNA extracted from virus grown in drug-treated cells sedimented as two distinct populations of 50S and 4S (Fig. 4A) (the identity of the small amount of 18S rRNA is uncertain), whereas virus produced by untreated cells contained, in addition, 28S and 18S RNAs (Fig. 4B). In the absence of proteinase K treatment before extraction, only small RNA species were ob-

tained. The fact that the 28S and 18S RNAs were inhibited by actinomycin D treatment strongly suggests that they were derived from host rRNA. The 4S RNA probably represents host tRNA. The virus preparations from which the RNA above was extracted (Fig. 4A and B) were not sonicated during purification. The amounts of 28S and 18S RNAs found associated with purified virions grown in untreated cells were reduced by about 80% as a result of sonication during the purification procedure, whereas 50S RNA was not affected by the sonication treatment (data not shown). This suggests that the bulk of the host RNA was probably not located inside virions but rather in the large vesicles (Fig. 1A) which copurify with virions.

Isolation of RS mRNA and annealing with virion RNA. Confluent HEp-2 cell monolayers were infected with RS virus at a multiplicity of 2 PFU per cell; mock-infected cultures served as uninfected controls. At 4 h p.i. all cultures were treated with 5 μg of actinomycin D per ml, and at 5 h 100 μCi of [5,6-^{3}H]uridine per ml was added to half of each of the infected and control cultures without removing the drug. At 23 h p.i. all cultures were harvested and extracted at pH 9.0 as described above. Figure 5 demonstrates the profiles of labeled control and infected cell RNA species resolved in 5 to 20% sucrose gradients. Infected cell cytoplasm at 23 h p.i. contained 50S, 35S, and a broad peak of 24S to 12S RNA species, whereas control cell cytoplasmic RNA preparations labeled under

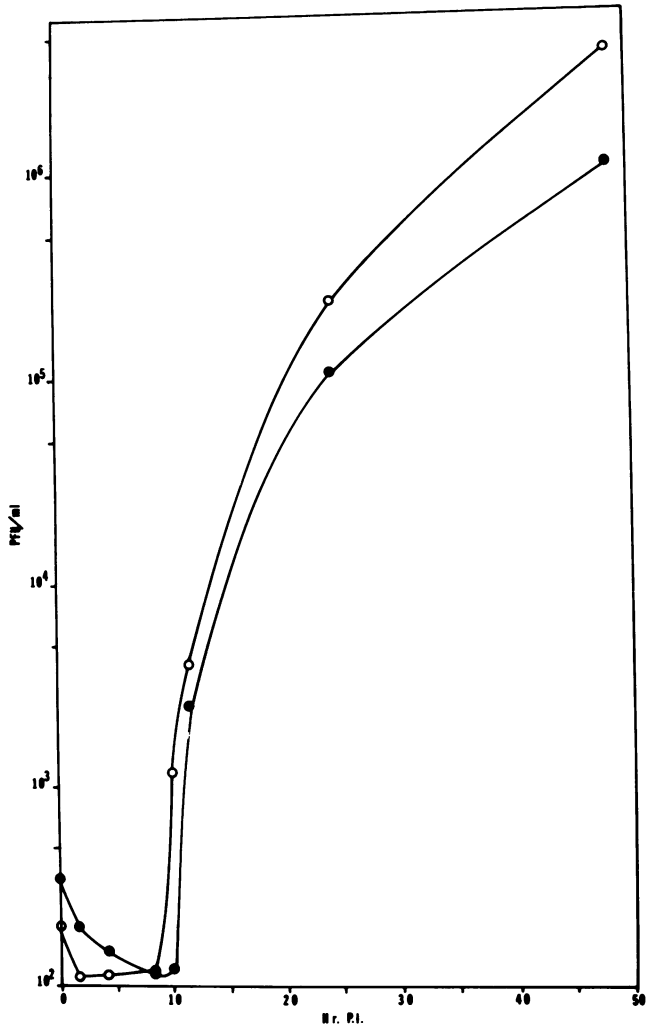

FIG. 3. *One-step growth curve of RS virus released into the medium by untreated (●) and actinomycin D-pretreated (0.5 μg/ml for 1 h before infection; ○) cells. Each time point represents an average of triplicate samples assayed for infectious virus using the methylcellulose overlay method described in the text.*

the same conditions contained only 12S material.

In similar experiments the four cultures (an unlabeled and a labeled infected culture and their control counterparts) were harvested; the RNA was extracted at pH 9.0 and then subjected to chromatography on oligo(dT)-cellulose columns, and the polyadenylated RNA was collected and concentrated by alcohol precipitation. The final recovery of polyadenylated RNA from infected versus control cells was in the ratio of 80:1. The presence of excessive amounts of free label in the extracts applied to the columns made it difficult to determine precisely the percentage of RNA bound to the columns. The unlabeled polyadenylated RNAs (see below for results with the labeled samples) were precipitated with alcohol, collected by centrifugation, and finally dissolved in 0.1 ml of water. Samples of these polyadenylated RNAs were mixed with 5 μl of [5-^{3}H]uridine-labeled 50S (obtained from a sucrose gradient) virion RNA also in water. The samples were annealed as described above, diluted in STE buffer, and tested for RNase sensitivity. Each sample was done in quadruplicate (Table 2).

Although the reactions were done in the presence of an excess of unlabeled polyadenylated or mRNA, the maximum amount of annealing achieved was 58.2%. The reasons for this are unclear and are the subject of a continuing investigation. We have determined that increasing (or decreasing) the incubation time does not increase the extent of annealing. It is unclear whether the polyadenosine tails are interfering with annealing or whether the mRNA samples taken at 23 h p.i. do not have a full complement of all the mRNA's encoded for by the viral genome.

It will be noted in Table 2 that as the final annealing volume is decreased the amount of annealing also decreases. The reasons for this

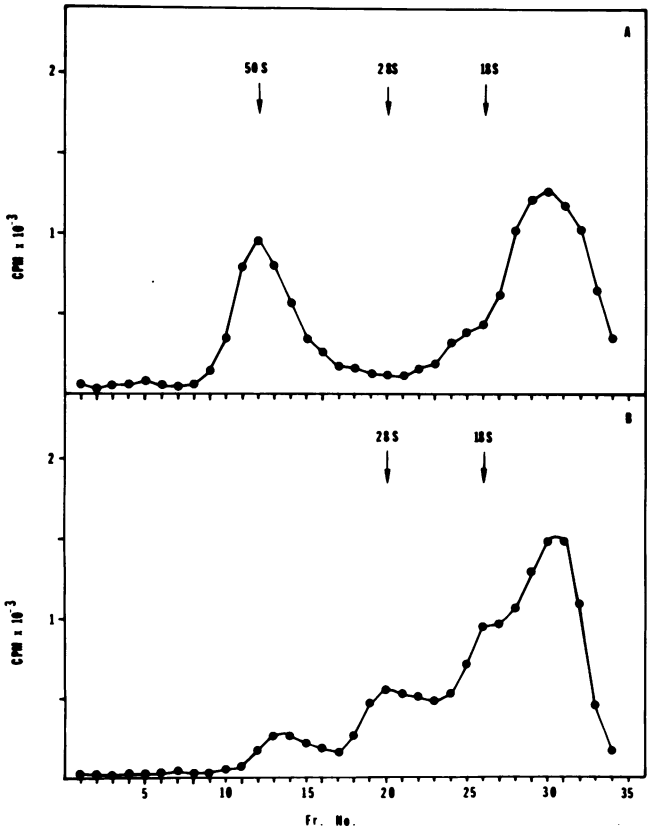

FIG. 4. *Sedimentation analysis of RNA extracted from virus grown in actinomycin D-treated or untreated HEp-2 cells. Virus was harvested at 72 h p.i. and purified, and the RNA was extracted. Centrifugation in 5 to 20% sucrose gradients was the same as described for Fig. 2B. (A) Sedimentation of RNA extracted from virus grown in actinomycin D-treated cells. (B) Sedimentation of RNA extracted from virus grown in untreated cells.*

apparent paradox are unclear, but based on these results and others involving the concentration-dependent behavior of the isolated viral 50S RNA during velocity sedimentation analyses, we feel it may be due to intermolecular aggregation. Apparently this RNA has a penchant for intermolecular aggregation. Again, this matter is also under continuing investigation.

Polyacrylamide gel electrophoresis of viral mRNA's. Labeled mRNA from infected cells was isolated by oligo(dT)-cellulose chromatography as described above. After overnight precipitation the RNA was treated to remove polyadenylate tracts and subjected to polyacrylamide gel electrophoresis as described in the text.

The results of such an analysis are shown in Fig. 6. The molecular weights of the RNAs were determined by calibrating the gels with influenza virus RNA segments using the values given by Desselberger and Palese (9) for unreduced RNAs. Genetic analysis has indicated that seven complementation groups exist, suggesting that seven primary gene products are made (13), whereas here only six RNA segments can be visualized. Whether one of the bands is a double band or whether the time of labeling and harvesting precludes seeing all RNAs is unknown and under investigation. If the isolated mRNA's are not subjected to annealing with oligo(dT) followed by digestion of the polyadenylate-oligo(dT) regions with RNase H, no mRNA's can be resolved by polyacrylamide gel electrophoresis. The gel pattern is one large smear. Indeed, the fuzziness of the RNA bands in Fig. 6 may be due to incomplete removal of the polyadenylate regions, although the presence of trace amounts of other RNases in the RNase H preparation has not been ruled out.

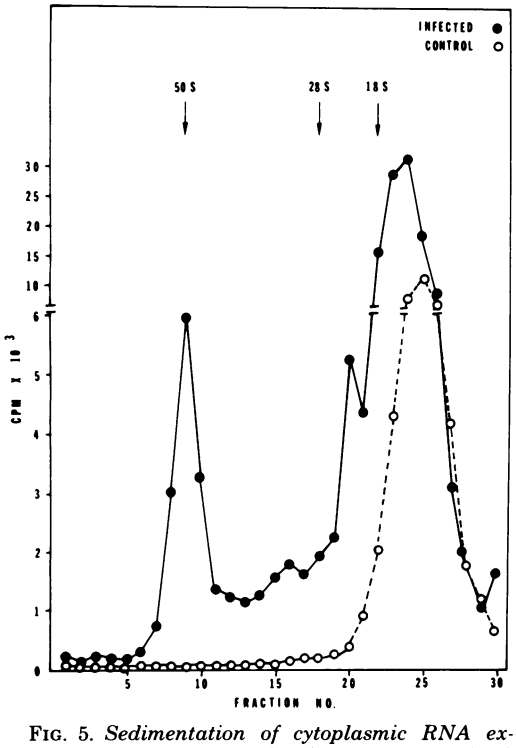

FIG. 5. *Sedimentation of cytoplasmic RNA extracted from control and RS-infected cells at 23 h p.i. in 5 to 20% sucrose gradients. Infected and control cells were treated with 5 μg of actinomycin D per ml at 4 h p.i., and at 5 h p.i. 100 μCi of [5,6-^{3}H]uridine per ml was added. Extracted RNA was centrifuged on 5 to 20% sucrose gradients at 17,000 rpm for 17 h in an SW27 rotor.*

TABLE 2. *Annealing of unlabeled RS virus mRNA to [^{3}H]uridine-labeled 50S virion RNA*

Annealing mixture (μl)[a]	Annealing vol (μl)[b]	cpm[b]		% Resistant
		With RNase	No RNase	
RS mRNA (20)	500	574	986	58.2
RS mRNA (10)	500	546	964	56.6
RS mRNA (1)	500	472	940	50.2
RS mRNA (0.1)	500	198	937	21.1
Control RNA (20)	500	126	930	13.6
No mRNA (self-annealed)	500	114	970	11.8
RS mRNA (20)	230	204	962	21.2
RS mRNA (20)	30	92	953	9.7

[a] RS mRNA and 5 μl of [^{3}H]uridine-labeled 50S viral RNA (approximately 2,000 cpm) in 2× SSC and 50% formamide were annealed at 50°C for 46 h. The details of the annealing procedure are given in the text.

[b] Values are the mean of four determinations.

DISCUSSION

Virus purification. The study of RS virus biochemistry has been hampered by the lack of an acceptable purification procedure which would allow both the isolation of highly pure virus and the efficient recovery of biologically active virions. Numerous attempts with a variety of techniques have been partially successful (18, 24, 25). We have presented in this report a purification scheme which fits both criteria mentioned above. We have routinely obtained 18 to 25% recovery of infectious virus, and by electron microscopic examination the final banded virus appears to be of high purity. It should be noted that the DNA-containing virus-like particle which we reported on elsewhere (Lambert and Pons, Abstr. Fourth Int. Congr. Virol. 1978, w24/2, p. 340) is not seen. Clearly, we were incorrect in that report; there is no DNA-containing virus-like particle in these preparations. Subsequent work showed that this was host-derived material which arose as a result of the extensive damage to the cell late in infection. The particles contained host DNA encapsidated within RS-specified protein envelopes (Lambert and Pons, un-

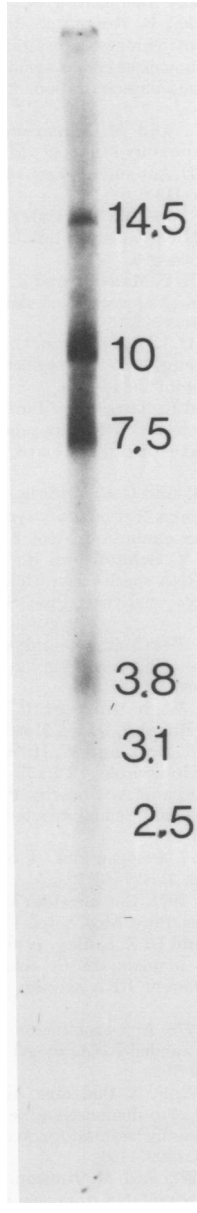

FIG. 6. *Polyacrylamide gel electrophoresis of [5,6-^{3}H]uridine-labeled RS mRNA's extracted from infected cells. Values are daltons* $\times 10^{-5}$.

published results).

An important finding during this study was that in addition to free RS virus particles, large vesicles containing virions were also observed during electron microscopic examination of virus preparations (Fig. 1A) that were harvested late in infection after syncytium formation was complete. It is obvious that such structures could contain a great deal of contaminating host material such as nucleic acids and proteins in addition to the virus particles. Sonication of this material during the purification procedure resulted in increased virus titers, presumably because of liberation of entrapped virions (Fig. 1B) and enhanced purity of virus (Table 1). It is possible that such structures arise as a result of budding of virus into cytoplasmic vesicles which are released from the cell as the viral cytopathic effect increases late in the infectious cycle.

Analysis of viral RNA. Viral RNA extracted from purified virus was found to consist of at least four separate size classes, the most abundant usually being 28S, 18S, and 4S (Fig. 2A and 4B). A relatively small amount of 50S RNA was consistently found, but only if the purified virus was treated with proteinase K before extraction. This suggests that some contaminating cellular RNase activity may have been associated with the virions. After treatment of infected cells with actinomycin D, purified virus contained only 50S and 4S RNA (Fig. 4A), suggesting that the 18S and 28S RNAs were ribosomal in origin since rRNA synthesis is readily inhibited by actinomycin D (20). The presence of rRNA in purified RS virus preparations was probably due to the harvesting of virus after extensive cytopathic effect had developed as has been reported for Sendai virus (17). The fact that about four times more 50S RNA was isolated from virus grown in actinomycin-treated cells than from virus grown in untreated cells (Fig. 4A and B) is due to stimulation of virus production in drug-treated cells (Fig. 3) (8, 23). The stimulatory effects of actinomycin D on RS virus synthesis appeared to be somewhat variable, and the amount of stimulation depended upon several parameters such as (i) the metabolic state of the tissue culture cells before infection, (ii) the multiplicity of infection, (iii) the concentration and length of drug treatment, and (iv) the time at which the drug was added after infection. The amount of stimulation of release of infectious virus in a typical experiment was on the order of 5- to 10-fold. The regimen of drug treatment described above was selected because it gave the most reproducible stimulation of infectious RS virus.

Sonication of virus grown in HEp-2 cells which were not treated with actinomycin D resulted in decreased amounts of 18S and 28S RNA associated with purified virions. While this suggests that the bulk of the rRNA's were present in the vesicles, it does not rule out that rRNA and tRNA molecules are also packaged within virions since they are still present after repeated banding of sonicated virus on linear sucrose gradients before extraction of the RNA.

Previous reports of high-molecular-weight RS

RNA have been made. Zhdanov et al. (27) reported the isolation of 52S RNA from virions and from infected cells. Hoffman et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, S300, p. 262) isolated a minor 50S RNA from viral ribonucleoprotein from infected cells and from RS virion ribonucleoprotein. A minor 50S RNA component has also been identified in RS infected BSC-1 cells and in virions (24). It is reasonable to assume that the 50S RNA represents the genomic RNA of RS virus. This size class of RNA is similar to that of other paramyxoviruses.

The demonstration of annealing of the 50S virion RNA to mRNA of infected cells but not control cells (Table 2) is the first positive evidence that RS virus is a negative stranded virus and lends support for its classification in the Paramyxoviridae group (16). The 50S RNA isolated from the cytoplasm of infected cells (Fig. 5) did not bind to oligo(dT), and probably represents, in part, full-length complementary RNA or newly synthesized 50S viral genomic RNA (data not shown). Six virus-specified mRNA species were resolved in cytoplasmic extracts of infected cells at 23 h p.i. by polyacrylamide gel electrophoresis (Fig. 6). Cash et al. (5) demonstrated that the in vitro translation of mRNA's from RS-infected BSC-1 cells resulted in the synthesis of six viral polypeptides, suggesting that at least six functional viral mRNA's were present in the cytoplasm of infected cells. Although obviously of a preliminary nature, the data on the isolation and characterization of the viral mRNA's indicate the strong similarities between RS virus and other paramyxoviruses.

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