JOURNAL OF VIROLOGY, Jan. 1977, p. 427-431

Vol. 21, No. 1
Printed in U.S.A.

Polypeptides of Respiratory Syncytial Virus

SEYMOUR LEVINE

Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, Michigan 48201

Received for publication 16 July 1976

Radiolabeled respiratory syncytial virus was purified from medium that had been harvested from infected HeLa cell monolayers before it contained much cellular debris. After isopycnic centrifugation in linear gradients prepared with sucrose dissolved in Hanks balanced salt solution, almost all the infectivity and most of the radioactivity were recovered in a single band with density from 1.16 to 1.23 g/cm^3 and a peak at 1.2 g/cm^3. Analysis by polyacrylamide gel electrophoresis resolved the purified virus into seven polypeptides of approximate molecular weights 20,000 to 80,000, of which the two largest and the smallest proved to be glycoproteins.

Respiratory syncytial (RS) virus, a major cause of disease in infants, resembles the paramyxoviruses in overall morphology, general appearance of the nucleocapsid, and maturation process, which involves budding from the surface of infected cells in an envelope derived from plasma membrane. However, RS virus differs from paramyxoviruses in not agglutinating erythrocytes and in particle size, nucleocapsid diameter, and the general appearance of intracytoplasmic inclusions produced by infection (1, 8).

Different paramyxoviruses have been reported to have from five polypeptides to as many as nine (2-4, 7, 9). There is uncertainty about some of those polypeptides, but there is apparent agreement that paramyxoviruses contain two glycoproteins whose molecular weights are 65,000 to 74,000 and 52,000 to 56,000, a nucleocapsid protein whose molecular weight is 55,000 to 60,000, and a nonglycosylated membrane protein with a molecular weight of 38,000 to 40,000.

Only one report has been published on the subunit composition of the RS virion. Wunner et al. found that partially purified virus contained five polypeptides whose molecular weight ranged from 20,000 to 61,000, including one glycoprotein (11). The paucity of information on subunits of RS virus is attributable to the problems involved in purifying it: its instability, the absence of a mechanism for shutting off cellular synthesis, and the continuing association of most of the produced virus with plasma membrane throughout the growth cycle. With the Long strain, over 90% of the virus produced is never released from the cell (6).

The Long strain virus used in our studies was grown and plaque assayed as previously described (5, 6) in HeLa cells maintained in continuous cultures and grown for virus assay or virus stocks as monolayers in 60- or 100-mm plastic petri dishes, in Eagle minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). To reduce the level of cellular components in the virus to be purified, we infected monolayers at a multiplicity of 3 to 5 PFU/cell, a multiplicity of infection high enough to infect most of the cells, to restrict virus production to a single cycle of growth, and we harvested the medium from infected cultures before it contained much cell debris. Before radiolabeling, the infected monolayers were incubated for 8 h in MEM supplemented with 5% FCS in an atmosphere of 5% CO_2 in air at 37°C. Then the medium was replaced with radiolabeled medium, which was harvested at ~24 h after infection and stored at −80°C. Prior to purification, the harvests were thawed and clarified by centrifugation at 5°C for 10 min at 1,000 × *g* in a model PRJ International centrifuge. The purification procedure is given in the legend to Fig. 1.

We found that when RS virus is diluted in water or diluted buffer, it loses ~90% of its infectivity in 18 h at 4°C, but it is stabilized by the divalent ions in Hanks balanced salt solution (HBSS) (Table 1). We therefore prepared sucrose for the density gradients used for purification in HBSS (sucrose-HBSS). Diluted in 51% sucrose-HBSS, the virus has a half-life at 4°C of ~48 h and is stable for at least one −80°C freeze-thaw cycle. By using sucrose-HBSS for the gradients, we preserved enough infectivity to follow the virus through the purification procedure.

After equilibrium centrifugation in a linear 40 to 60% sucrose-HBSS gradient, the last step in purification, 1-ml fractions were collected with a Buchler Densiflow apparatus, starting from the top of the gradient, and were sampled for infectivity, for scintillation counting, and to determine refractive index in a Bausch and Lomb refractometer. Fractions containing virus were pooled, diluted to less than 20% sucrose with cold HBSS, and centrifuged for 5 h through 20% sucrose-HBSS. The purified virus pellet was dissociated in 0.005 M sodium phosphate buffer (pH 7.2) containing 1% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol by immersion in boiling water for 2 min. Samples were then subjected to polyacrylamide gel electrophoresis, as described in the legend to Fig. 2 (A).

For scintillation counting of liquid samples, 0.1-ml samples were mixed with 0.1 ml of 1 N NaOH and hydrolyzed for 2 h at 50°C. Then 0.8 ml of 0.1 N HCl and 10 ml of a toluene-Triton X-100 scintillation cocktail were added to each vial. For counting polyacrylamide gels, they

TABLE 1. *Effect of HBSS or Mg^{2+} and Ca^{2+} on stability of respiratory syncytial virus when stored at 4°C for 18 h*

Virus prepn	PFU/ml[a]
Undiluted virus stock	3.8×10^6
Diluted 1:30 in HBSS	5.5×10^6
Diluted 1:30 in phosphate-buffered NaCl[b]	0.26×10^6
Diluted 1:30 in phosphate-buffered NaCl + $MgSO_4$ + $CaCl_2$[c]	2.4×10^6

[a] Calculated to the concentration present in the undiluted virus.

[b] Phosphate buffer (pH 7.2) at 0.001 M, the concentration of phosphate in HBSS, plus 0.85% NaCl.

[c] $MgSO_4$ at 0.008 M plus $CaCl_2$, at 0.002 M, the concentration of these salts in HBSS.

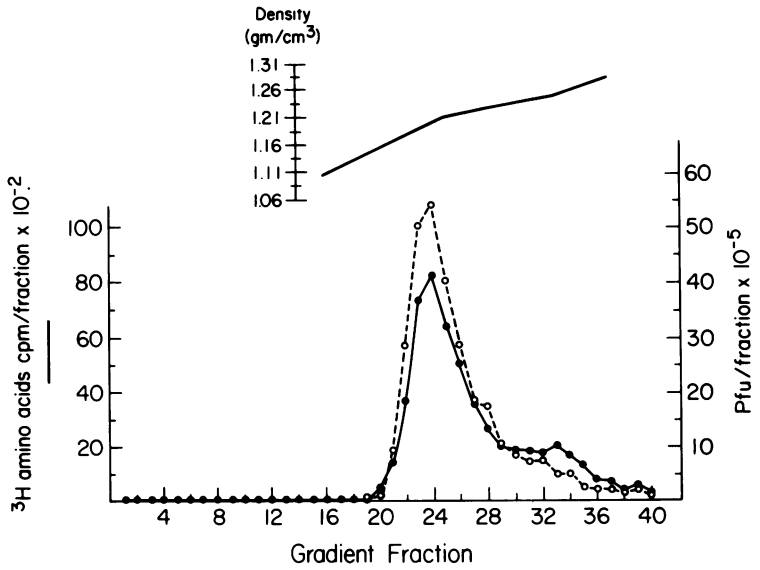

FIG. 1. *Isopycnic centrifugation in a linear sucrose-HBSS gradient of ^{3}H-amino acid-labeled RS virus collected from the 35-60 sucrose-HBSS interface. (Migration in gradient is from left to right.) After incubation of the infected monolayers for 8 h, their medium was replaced with MEM containing 50% of its normal concentration of amino acids and supplemented with 5% FCS and 5 μCi of ^{3}H-labeled reconstituted protein hydrolysate (Schwarz/Mann) per ml. That medium was harvested for virus purification 22 to 24 h after infection, when monolayers had developed numerous syncytia but before the medium contained much cellular material, and was stored at −80°C in a Revco freezer pending purification, as was the virus material between steps of the purification procedure. After the initial centrifugation for clarification of the virus material, all centrifugation was at 5°C in an SW27 rotor at 27,000 rpm. The clarified supernatant was centrifuged for 4 h through 20% sucrose prepared in HBSS onto a cushion of 60% sucrose-HBSS. The band from that 20-60 interface was diluted in cold HBSS and centrifuged for 7 h through 35% sucrose-HBSS onto a 60% sucrose-HBSS cushion. The band from that 35-60 interface was diluted in cold HBSS to less than 30% sucrose and centrifuged to equilibrium (for 44 to 48 h) on a linear 40 to 60% sucrose-HBSS gradient. Then fractions were collected and sampled for subsequent procedures as described in the text.*

were frozen at −20°C, cut into 2-mm slices with stacked razor blades, and placed in counting vials along with 0.2 ml of 1 N NaOH. The vials were incubated for 2 h at 50°C and stored overnight at room temperature and, to each, 0.6 ml of 0.2 N HCl and 10 ml of the Triton X-100 cocktail were added. Before counting in a Packard model 2420 Tri-Carb liquid scintillation counter, all vials were stored at room temperature until counts had stabilized.

In a curve for the distribution of radiolabel and infectivity in the isopycnic linear sucrose gradient, both activities migrated together in a single major band, peaking at a density of 1.2 g/cm^3 (Fig. 1). Fractions 21 to 31, densities 1.16 to 1.23, were pooled, pelleted through 20% sucrose, dissociated with SDS, and subjected to polyacrylamide gel electrophoresis. The resulting pattern had five distinct peaks of radiolabel, polypeptides no. 1 to 5, and a faster-migrating, relatively broad, minor peak, no. 7 (Fig. 2A). Polypeptide no. 6 migrated as either a peak or a shoulder associated with the advancing boundary of no. 5. The average molecular weights of the virion polypeptides were determined by the method of Weber and Osborn (10), using as standards phosphorylase a, bovine serum albumin, ovalbumin, pepsin, and α-chymotrypsinogen A, all of which were obtained from Sigma. The average molecular weights of the seven polypeptides, based on the results obtained in five separate electrophoresis runs with marker proteins, were 79,000, 56,000, 44,000, 32,000, 28,000, 25,000, and 22,000. The values for no. 6 and 7 may be less reliable than the others, because the smallest marker protein used has a molecular weight of 25,7000.

With RS virus, which has no mechanism for shutting off cellular synthesis, it is difficult to determine whether any polypeptides in the purified virus are cellular contaminants, since some cellular polypeptide synthesis takes place throughout the viral growth cycle. There was not enough cellular material in medium incubated for 24 h with noninfected monolayers to use that for specifying the cellular contaminants in purified virus. By carrying lysates of noninfected, radiolabeled cells through the virus purification procedure, we were able to eliminate those components that would copurify with virus because they were the same size and density (Fig. 2B). This material was apparently composed of many overlapping polypeptides, which suggests that all the polypeptides found in the virus preparation, with the possible exception of no. 7, were viral, not cellular. However, no. 7 is evidently a glycoprotein (see below) and appears to be one of the glucosamine-labeled polypeptides present in infected cells but not in noninfected cells (S. Levine and M. Peeples, submitted for publication). Although we have not yet resolved the problem of cell contamination closely associated with the virus during purification, the presence of only seven discrete polypeptides in the virus preparations makes it very likely that these are indeed virion polypeptides.

To identify the virion glycoproteins, virus labeled with [^{14}C]glucosamine was purified, dissociated with SDS, mixed with virus labeled with ^{3}H-amino acids, and subjected to co-electrophoresis. The two largest polypeptides and no. 7 contained the glucosamine and so must have been glycoproteins (Fig. 2C). The very low levels of glucosamine (<50 counts/min) associated with the other viral polypeptides could have been either background or a low level of cellular contamination, since glucosamine-labeled infected and noninfected cells have many overlapping glycoproteins (S. Levine, unpublished observations).

Although the assumption that the released virus we used for purification also represents the cell-associated virus is reasonable, it cannot be tested until we have a method for separating the unreleased virus without losing infectivity. Preliminary experiments suggest that a noninfectious particle that bands at density 1.14 to 1.17 g/cm^3, with a peak at 1.16, can be isolated from ^{3}H-amino acid-labeled lysates of cells treated with trypsin. On polyacrylamide gels, this fraction from the gradient contains polypeptides no. 3, 4, 5 and 6 and a reduced level of no. 2. This suggests that polypeptides no. 2 to 6 are present in the unreleased virus and that the absence of no. 1 and 7, like the reduced quantity of no. 2, results from their digestion by the trypsin.

The polypeptide composition of RS virus apparently differs from that of the paramyxoviruses. The molecular weight of the two major RS virion glycoproteins, 79,000 and 56,000, are close to the molecular weight of the two paramyxovirus glycoproteins, but the other polypeptides, which include a third glycoprotein and presumably also nucleocapsid and membrane proteins, are all smaller than those of the paramyxoviruses.

I gratefully acknowledge the able technical assistance of Bruce Linebaugh and Mei Cheng and the editorial assistance of Esther Levine.

This work was supported by Public Health Service research grant AI-10996 from the National Institute of Allergy and Infectious Diseases.

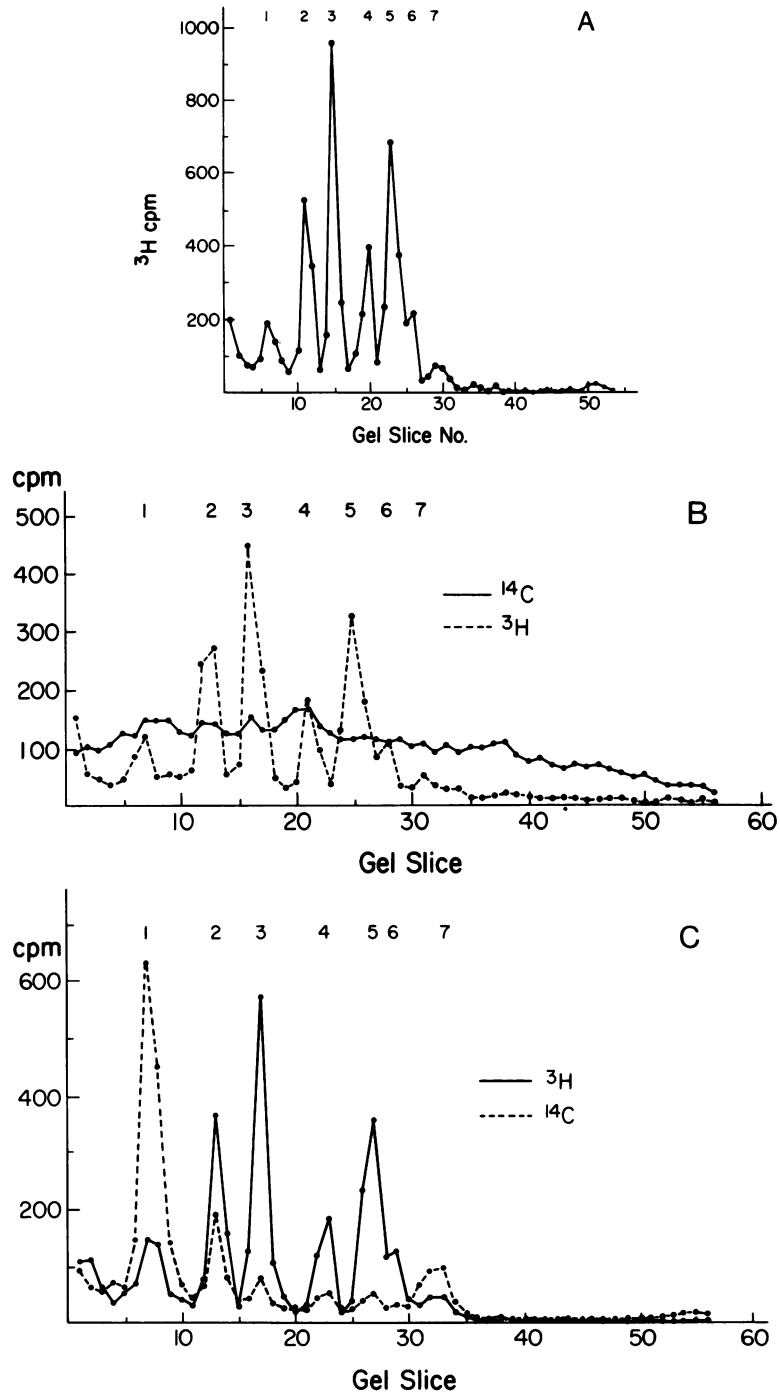

FIG. 2. *(A) SDS-polyacrylamide gel electrophoresis of ^{3}H-amino acid-labeled RS virus collected from the isopycnic gradient. Samples of SDS-β-mercaptoethanol-dissociated, purified virus (50 or 100 μl, containing 5,000 to 10,000 counts/min) were subjected to electrophoresis on 10-cm gels containing 10% polyacrylamide and 0.1% SDS, with a 0.5-cm stacking gel containing 2.5% polyacrylamide. The electrode buffer was 0.1 M phosphate containing 0.1% SDS. Gels were prerun for 2 h at 4 mA/gel. Samples were run for 16 to 18 h at 2 mA/gel and then at 8 mA/gel until the tracking dye reached the bottom of the gel (2 to 3 h). (B) Co-electrophoresis on SDS-polyacrylamide gel of purified, ^{3}H-amino acid-labeled RS virus and "virus-like"*

LITERATURE CITED

1. **Berthiaume, L., J. Joncas, and V. Pavilanis.** 1974. Comparative structure, morphogenesis and biological characteristics of respiratory syncytial (RS) virus and pneumonia virus of mice (PVM). Arch. Gesamte Virusforsch. 45:39–51.
2. **Hall, W. W., and S. J. Martin.** 1973. Purification and characterization of measles virus. J. Gen. Virol. 19:175–188.
3. **Iinuma, M., and R. W. Simpson.** 1974. The structural proteins of Newcastle disease virus: identification of a minor internal protein. Virology 61:229–239.
4. **Jensik, S. C., and S. Silver.** 1976. Polypeptides of mumps virus. J. Virol. 17:363–373.
5. **Levine, S., D. A. Buthala, and R. D. Hamilton.** 1971. Late stage synchronization of respiratory syncytial virus replication. Virology 45:390–400.
6. **Levine, S., and R. Hamilton.** 1969. Kinetics of the respiratory syncytial virus growth cycle in HeLa cells. Arch. Gesamte Virusforsch. 28:122–132.
7. **Mountcastle, W. E., R. W. Compans, and P. W. Choppin.** 1971. Proteins and glycoproteins of paramyxoviruses: a comparison of simian virus 5, Newcastle disease virus, and Sendai virus. J. Virol. 2:47–52.
8. **Norrby, E., H. Marusyk, and C. Orvell.** 1970. Morphogenesis of respiratory syncytial virus in a green monkey kidney cell line (Vero). J. Virol. 6:237–242.
9. **Waters, D. J., and R. H. Bussel.** 1973. Polypeptide composition of measles and canine distemper virus. Virology 55:554–557.
10. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406–4412.
11. **Wunner, W. H., G. P. Faulkner, and C. R. Pringle.** 1975. Respiratory syncytial virus: some biological and biochemical properties, p. 193–201. *In* B. W. J. Mahy and R. D. Barry (ed.), Negative strand viruses. Academic Press Inc., New York.

fraction from ^{14}C-amino acid-labeled, noninfected cells. Monolayers were labeled with ^{14}C-labeled reconstituted protein hydrolysate (Schwarz/Mann) between 8 and 24 h after "mock infection" and disrupted by one freeze-thaw cycle, and the cell lysates were carried through the virus purification procedure. Fractions were collected from the isopycnic density gradient covering the range of densities collected from the virus gradients and pelleted through 20% sucrose-HBSS, and the pellet was dissociated with SDS and β-mercaptoethanol. A sample of this noninfected preparation was co-electrophoresed with the ^{3}H-amino acid-labeled virus. (C) Co-electrophoresis on SDS-polyacrylamide gel of purified, ^{3}H-amino acid-labeled RS virus and purified, [^{14}C]glucosamine-labeled RS virus. After incubation of infected monolayers, their medium was replaced with MEM plus 5% FCS containing 4 μCi of D-[1-^{14}C]glucosamine-hydrochloride (specific activity, 56.5 mCi/ml) per ml obtained from ICN. After purification and dissociation, the glucosamine-labeled virus was mixed with the amino acid-labeled virus, and they were subjected to co-electrophoresis on 10% polyacrylamide.