

## Analysis of Proteins Synthesized in Respiratory Syncytial Virus-Infected Cells

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Received 7 October 1981/Accepted 4 January 1982

The spectrum of respiratory syncytial virus-encoded proteins was examined in infected cell extracts by standard polyacrylamide gel electrophoresis and by two-dimensional gel analysis. Polyacrylamide gel electrophoresis analysis of a variety of respiratory syncytial virus-infected, actinomycin D-treated cell lines revealed the presence of as many as nine virus-encoded proteins. Seven of these nine proteins were immunoprecipitated by anti-respiratory syncytial serum. Only one major band of [<sup>3</sup>H]glucosamine was detected in infected cell extracts (Vp86), whereas the reported major virion glycoprotein (Vp48-53) was difficult to detect in infected cells when carbohydrate labels were employed. Two-dimensional gel analysis easily identified seven viral proteins, and one other was tentatively identified. The reported major virion glycoprotein again was not consistently detected. The results of this study confirm the existence of a virus-coded glycoprotein (Vp86) in infected cell extracts. The existence of this glycoprotein in the purified virion has been in dispute, but the apparent low methionine content of this protein may be the reason for this controversy.

Respiratory syncytial (RS) virus is the major cause of lower respiratory tract illnesses during infancy. Despite the recognition of this agent as a major human pathogen, neither successful preventive nor effective therapeutic strategies have been developed to reduce the incidence of this virus-induced disease. Recently, we identified several low-molecular-weight amidine-containing compounds which interfere with the biological activity of RS virus *in vitro* and *in vivo* (7, 8; unpublished data). The mechanism by which these compounds exert their effect is unknown. One possibility is that amidines interfere with the synthesis or processing of RS virus proteins. Of particular interest are the viral proteins which are found on the surface of infected cells, since the amidine inhibitors may disrupt normal host cell-virus interactions. This study was undertaken to define the spectrum of RS virus-induced proteins in infected cells to ascertain the impact of amidine inhibitors on viral protein synthesis. The analysis was achieved by standard polyacrylamide gel electrophoresis (PAGE), two-dimensional PAGE, and immunoprecipitation.

### MATERIALS AND METHODS

**Viruses and cells.** The A<sub>2</sub> and Long strains of RS virus were obtained from R. M. Chanock (National

Institute of Allergy and Infectious Diseases, Bethesda, Md.). Viral stocks were prepared in HEp-2 cell cultures propagated in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. All cell lines (HEp-2, LLC-MK<sub>2</sub>, CV-1, and BS-C-1) were obtained from Flow Laboratories, Inc., McLean, Va. Viral stocks and cell lines were determined to be free of mycoplasma contamination by the fluorochrome technique of Chen (5). Extracts of cells grown in the absence of antibiotics were incubated with mycoplasma-free Vero cells for 5 days before fluorochrome staining.

**Isotopic labeling of virus-specific proteins.** Cells used for the labeling experiments were grown as monolayers in 24-well trays (Falcon Plastics, Oxnard, Calif.). RS virus (A<sub>2</sub> strain) was added at an input multiplicity of infection of 5 to 10. At 6 h postinfection, the medium was removed and replaced with MEM plus 5% fetal bovine serum containing 2.5 μg of actinomycin D per ml. Viral proteins were labeled at 20 to 24 h postinfection with either 50 μCi of [<sup>35</sup>S]methionine in 250 μl of methionine-free MEM or 50 μCi of [<sup>3</sup>H]leucine in 250 μl of leucine-free MEM. Viral glycoproteins were labeled with either 25 μCi of [<sup>3</sup>H]glucosamine or 50 μCi of [<sup>3</sup>H]mannose in 250 μl of 1:1 MEM-phosphate-buffered saline (PBS).

Cell extracts for one-dimensional PAGE were prepared by solubilizing the cellular contents of a single well in 400 μl of electrophoresis sample buffer (62.5 mM Tris-hydrochloride, pH 6.8; 2% sodium dodecyl sulfate (SDS); 20% glycerol; 2% 2-mercaptoethanol). Samples were heated at 95°C for 2 to 4 min and frozen at -70°C. Extracts for nonequilibrium pH gradient electrophoresis (NEPHGE) and isoelectric focusing (IEF) were prepared as follows. Cell monolayers were

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rinsed two times with PBS and then scraped into 400  $\mu$ l of PBS. The cells were pelleted in a microcentrifuge tube and then solubilized with 110  $\mu$ l of 0.5% Nonidet P-40 (NP-40) in water. The extracts were mixed in a Vortex mixer and maintained at room temperature for 10 min. The nuclei were pelleted, and 100  $\mu$ l of supernatant was added to 135 mg of urea. This was followed by the addition of 7  $\mu$ l of 20% SDS, 13  $\mu$ l of ampholines (pH 3.5 to 10), 13  $\mu$ l of 2-mercaptoethanol, and 20  $\mu$ l of NP-40. Extracts were frozen at  $-70^{\circ}\text{C}$ .

**Immunoprecipitation.** Cell extracts for immunoprecipitation were solubilized in 0.5% NP-40 in PBS. Antibody to RS virus was purchased from Flow Laboratories. Since this antiserum was prepared with RS virus grown in HEP-2 cells, all extracts for immunoprecipitation were prepared using CV-1 cells or LLC-MK<sub>2</sub> cells. Immunoprecipitates were prepared by the procedure of Hayes et al. (9), with Formalin-fixed *Staphylococcus aureus* (Cowan 1 strain).

**One-dimensional PAGE.** Slab gels (100 by 140 by 1.5 mm) consisted of 10% acrylamide (0.27% bisacrylamide), 12.5% acrylamide (0.14% bisacrylamide), or 15% acrylamide (0.086% bisacrylamide). The discontinuous buffer system of Laemmli and Favre (10) was used with a 3.5% acrylamide stacking gel. Molecular weight estimations of RS virus proteins were made with either the proteins of vesicular stomatitis virus (G, 69,000; N, 50,000; and M, 29,000) (19) or marker proteins from Sigma Chemical Co., St. Louis, Mo. (bovine albumin, 66,000; egg white albumin, 45,000; trypsinogen, 24,000; lactoglobulin, 18,400; and lysozyme, 14,300) as markers. Linear regression analysis was used to estimate molecular weights.

**Two-dimensional PAGE.** First-dimension IEF gels (ampholines, pH 5 to 7) were prepared according to O'Farrell (14), and NEPHGE (ampholines, pH 3.5 to 10) was performed as described by O'Farrell et al. (15). Electrode solutions were 0.02 M NaOH and 0.01 M phosphoric acid. For IEF, electrophoresis was for 18 h at 350 V followed by 1 h at 700 V; for NEPHGE, gels were run at 500 V for 5 h. First-dimension cylindrical gels (2.5 mm) were extruded into tubes containing sample buffer and were frozen at  $-70^{\circ}\text{C}$ .

Two-dimensional PAGE was performed as described by Dubovi and Wagner (8). First-dimension gels were equilibrated for 1 h at  $37^{\circ}\text{C}$  in sample buffer and then immobilized on top of slab gels with 1% agarose. Slab gels were analyzed by autoradiography for  $^{35}\text{S}$  and fluorography for  $^{35}\text{S}$  or  $^3\text{H}$  (2) with Kodak X-OMAT XRP-5 film.

**Chemicals and isotopes.** Reagents for PAGE were purchased from Bio-Rad Laboratories, Richmond, Calif. Isotopes L-[ $^{35}\text{S}$ ]methionine (890 to 1,110 Ci/mmol), D-[6- $^3\text{H}$ ]glucosamine hydrochloride (30 Ci/mmol), and D-[2- $^3\text{H}$ ]mannose (16 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, Ill., and L-[4,5- $^3\text{H}$ ]leucine (56.8 Ci/mmol) was purchased from ICN Pharmaceuticals, Irvine, Calif. Ampholines were obtained from LKB Instruments Inc., Rockville, Md., and actinomycin D was from Sigma.

## RESULTS

**Labeling of RS virus-infected cells with [ $^{35}\text{S}$ ]methionine.** The initial set of experiments was designed to explore conditions which would provide acceptable labeling of RS virus proteins

in infected cells. Since RS virus infection does not lead to a rapid shutoff of host cell protein synthesis (12), actinomycin D was added to infected cells at various times, and viral proteins were labeled with [ $^{35}\text{S}$ ]methionine 18 to 24 h postinfection. One-dimensional PAGE of RS virus-infected CV-1 cell extracts showed the presence of at least eight new polypeptides (Vp68, Vp50, Vp45, Vp36, Vp33, Vp24, Vp18, and Vp15 [Fig. 1 and 2]). These same polypeptides were labeled with [ $^{35}\text{S}$ ]methionine in RS virus-infected Vero, BS-C-1, LLC-MK<sub>2</sub>, A549, HEP-2, and BHK-21 cell lines.

**Labeling of RS virus-infected cells with [ $^3\text{H}$ ]glucosamine.** RS virus-infected CV-1 and LLC-MK<sub>2</sub> cells were labeled with [ $^3\text{H}$ ]glucosamine for 3 h, beginning 24 h postinfection. In both cell lines, one major band of protein was labeled with [ $^3\text{H}$ ]glucosamine (Fig. 1). This band did not coincide with any [ $^{35}\text{S}$ ]methionine-labeled polypeptide. PAGE analysis of a mixture of [ $^3\text{H}$ ]glucosamine- and [ $^{35}\text{S}$ ]methionine-labeled cell extracts confirmed the uniqueness of the protein designated Vp86 (data not shown). Longer labeling times with [ $^3\text{H}$ ]glucosamine or [ $^3\text{H}$ ]mannose did not alter the pattern from that shown in Fig. 1. Longer development times of the fluorograms revealed minor bands of carbohydrate label in the areas of Vp50 and Vp24. Trichloroacetic acid precipitates of supernatant fluids from infected cultures labeled for 20 h (6 to 26 h

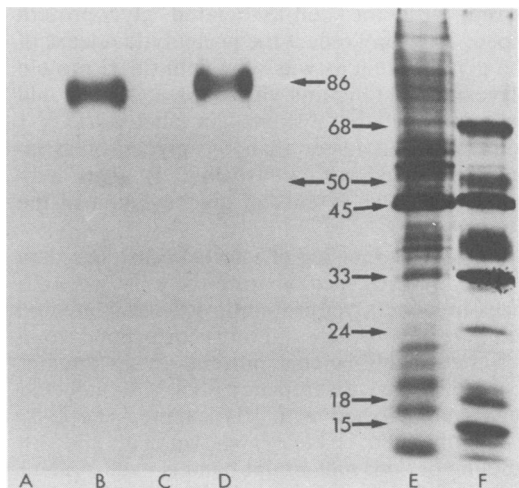


FIG. 1. SDS-PAGE of [ $^3\text{H}$ ]glucosamine-labeled RS virus-infected CV-1 (lanes A and B) and LLC-MK<sub>2</sub> (lanes C and D) cells. Actinomycin D-treated, control (lanes A and C), and RS virus infected cells (lanes B and D), were labeled with [ $^3\text{H}$ ]glucosamine for 3 h, beginning 24 h postinfection. Gels were processed for fluorography (2). Lanes E and F are CV-1 control and infected cell extracts labeled with [ $^{35}\text{S}$ ]methionine, respectively. The spaces between lanes D and E were blank wells. Gel concentration, 12.5%.

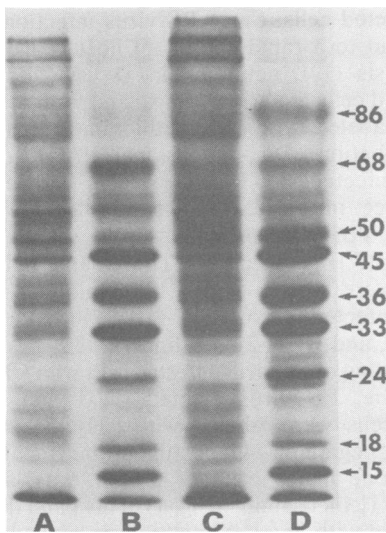


FIG. 2. SDS-PAGE of RS virus-infected LLC-MK<sub>2</sub> cells. Actinomycin D-treated cells, LLC-MK<sub>2</sub> control (lanes A and C) and RS virus-infected (lanes B and D), were labeled with [<sup>3</sup>H]leucine for 1 h (lanes A and B) or 3 h (lanes C and D), beginning 24 h postinfection. Gel concentration, 12.5%.

postinfection) with [<sup>3</sup>H]glucosamine also showed only one major band of carbohydrate label (data not shown). In this instance, the molecular weight was reduced slightly as compared with the cell-associated glycoprotein Vp86. This may reflect the proteolytic release of the glycoprotein as was seen with the G protein of vesicular stomatitis virus (13). It also should be noted that the glycoprotein labeled in CV-1 cells migrated faster than the glycoprotein labeled in LLC-MK<sub>2</sub> cells (Fig. 1). This may reflect different extents of glycosylation in the two cell lines.

**[<sup>3</sup>H]leucine labeling of infected cells.** The data in Fig. 1 clearly demonstrate the existence of a virus-induced glycoprotein in RS virus-infected cell extracts which did not correspond to a [<sup>35</sup>S]methionine-labeled protein. In an attempt to resolve this discrepancy, RS virus-infected cells were labeled with [<sup>3</sup>H]leucine. The existence of Vp86 is clearly shown with the 3-h labeling period, and a faint band is even evident with a 1-h labeling period (Fig. 2). Labeling times as long as 20 h with [<sup>35</sup>S]methionine failed to demonstrate this polypeptide. Since [<sup>35</sup>S]methionine has been used for many of the studies of RS virus proteins, this could explain the inability of these workers to detect a Vp86 glycoprotein in RS virus-infected cells (3, 4, 17, 20).

**Immunoprecipitation of labeled proteins.** The detection of new protein bands in virus-infected cells strongly suggests a viral origin for these

proteins. Additional support for this supposition is achieved by demonstrating that these proteins are immunologically related to virion proteins. To this end, RS virus-infected LLC-MK<sub>2</sub> cell extracts were reacted with a horse anti-RS virus serum raised against virus grown in Hep-2 cells. Analysis of the immunoprecipitates indicated that at least Vp86, Vp68, Vp50, Vp45, Vp36, Vp33, and Vp24 are of viral origin (Fig. 3). The low activity toward Vp18 and Vp15 is not surprising since these proteins have not been detected in the virion (4, 11, 16, 17). Sera from cotton rats productively infected with RS virus also showed a similar pattern of immunoprecipitation. Finally, [<sup>3</sup>H]glucosamine-labeled Vp86 also specifically reacted with anti-RS serum (data not shown).

**Two-dimensional gel analysis of RS virus-infected cells.** By standard PAGE, nine RS virus-induced proteins were identified in a variety of infected cell extracts (Fig. 1 and 2). Confirmation of these results was attempted by employing

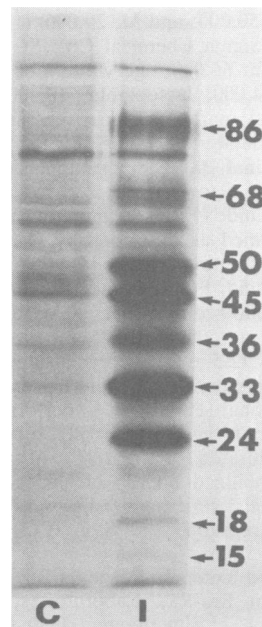


FIG. 3. Immunoprecipitation of [<sup>3</sup>H]leucine-labeled proteins from RS virus-infected LLC-MK<sub>2</sub> cells. RS virus-infected LLC-MK<sub>2</sub> cells were labeled for 3 h, beginning 24 h postinfection, with [<sup>3</sup>H]leucine. Cells were solubilized with 0.5% NP-40 in PBS. Extracts that were preabsorbed with Formalin-fixed *S. aureus* (Cowan 1 strain) were incubated with horse anti-RS serum (Flow Laboratories) for 1 h at room temperature. Antigen-antibody complexes were collected using the staphylococcal A protein binding technique (9). Pelleted complexes were washed three times and then dissolved in electrophoresis sample buffer. Gel concentration, 12.5%.

NEPHGE (15) and IEF (14) gels in a two-dimensional analysis of RS virus-infected cell extracts. Positive identification of seven viral proteins was made by NEPHGE gel analysis (Fig. 4). Proteins Vp86, Vp45, Vp36, Vp33, Vp24, Vp18, and Vp15 were easily identified with the [<sup>3</sup>H]leucine label, whereas Vp86 was absent with a [<sup>35</sup>S]methionine label. Protein Vp68 was difficult to identify in all samples, but a tentative location is indicated in Fig. 4. Protein Vp50 also was not positively identified, but a band is occasionally seen as in Fig. 4 in the basic region of the gel above the Vp45 streak. A protein of this molecular weight (VGP48) was identified as a virion glycoprotein (4, 11, 16, 17), but its existence in infected cells is difficult to confirm (4). The same viral proteins shown in Fig. 4 were identified with RS virus-infected HEp-2 cell extracts labeled in the absence of actinomycin D (data not shown).

The NEPHGE gel analysis also provides information on the acidic or basic nature of the viral proteins. Clearly, Vp86 and Vp36 are acidic, whereas Vp33, Vp24, and Vp18 are basic. Protein Vp45 was not resolved into a single band by this technique. This may be because this protein, which in all probability is the nucleocapsid protein (20), may be tightly associated with the RNA and the solubilization procedure for NEPHGE gels did not fully disrupt this interaction. Protein Vp36, which can run as a diffuse band on standard gels, also presents a unique profile on two-dimensional gels. The large trailing spot in Fig. 4 was characteristic of Vp36 on the second-dimension slab gel.

Since the existence of Vp86-like polypeptides has been in question, infected cell extracts were subjected to IEF to clearly separate Vp86 from other acidic proteins which might remain at the origin of NEPHGE gels. These gels show that no host cell proteins migrate to a position similar to that of Vp86 (Fig. 5). Viral protein Vp36 also is resolved at the acidic end of the gel, whereas Vp15 is somewhat less acidic. The basic proteins (Vp68, Vp33, Vp24, and Vp18) did not migrate from the origin, which was excluded from this photograph.

## DISCUSSION

This study was initiated in response to the recent findings that amidine-containing compounds specifically interfere with RS virus-host cell interactions (6, 7). To ascertain the mechanism by which these compounds inhibit RS virus, it is necessary to identify the RS virus-specific proteins found in and on infected cells. The data presented in this report argue strongly for the existence in infected cells of at least nine RS virus-specific polypeptides (Vp86, Vp68,

Vp50, Vp45, Vp36, Vp33, Vp27, Vp18, and Vp15). This spectrum of viral proteins was detected in at least six different cell lines. A 10th protein with a molecular weight similar to that of the L protein of vesicular stomatitis virus (Vp190) was inconsistently detected in RS virus-infected cells (data not shown). This protein probably corresponds to the Vp200 protein detected by others (4, 17).

Of the nine proteins identified in this work, seven were immunoprecipitable with RS virus-specific antiserum (Vp86, Vp68, Vp50, Vp45, Vp36, Vp33, and Vp27 [Fig. 3]). At present, there is still some doubt expressed as to the viral nature of a glycoprotein with a molecular weight similar to that of Vp86 (17). Bernstein and Hruska (1) also found an immunoprecipitable glycoprotein similar to Vp86 in RS virus-infected cells; however, Pringle et al. (17) could not confirm its viral origin, even though a specific association of this polypeptide with a productive RS virus infection was established. Glycoprotein Vp86 was the major protein detected in RS virus-infected cell extracts of LLC-MK<sub>2</sub> and CV-1 cells when a [<sup>3</sup>H]glucosamine label was employed (Fig. 1). In addition, supernatant fluids from these cells contained a single glycoprotein with a slightly lower molecular weight than that of Vp86. Both [<sup>3</sup>H]leucine-labeled Vp86 and [<sup>3</sup>H]glucosamine-labeled Vp86 were immunoprecipitable. Two-dimensional gel analysis of control and RS virus-infected cell extracts (Fig. 4 and 5) confirmed that Vp86 existed only in infected cells. These data taken together argue strongly for the viral origin of Vp86.

Part of the problem in identifying Vp86 as a viral protein may lie in the difficulty in labeling this protein with [<sup>35</sup>S]methionine. Although [<sup>3</sup>H]glucosamine easily labeled this polypeptide, [<sup>35</sup>S]methionine incorporation into Vp86 is very limited (Fig. 1) (1, 17). However, Vp86 is easily labeled with [<sup>3</sup>H]leucine (Fig. 2) or a mixture of <sup>3</sup>H-amino acids (11). This labeling pattern suggests that Vp86 is not a precursor to the reported major virion glycoprotein VGP48 since this protein is labeled with [<sup>35</sup>S]methionine.

The data in Fig. 3 also confirm the viral origin of Vp68. Bernstein and Hruska (1) detected a similar polypeptide (Vp73) but were not able to show immunoprecipitation with RS virus-specific rabbit antiserum. Ueba (18) also found a comparable protein (Vp75) in purified virus, but others have not reported the existence of this polypeptide (3, 4, 11, 16, 17, 20). The data in Fig. 2 demonstrate a consistent finding regarding Vp68; i.e., more label is found in Vp68 in a 1-h labeling period than in a 3-h labeling period. In addition, pulse-chase experiments show a decrease in Vp68 during the chase period (data not shown). These data suggest that Vp68 might be a

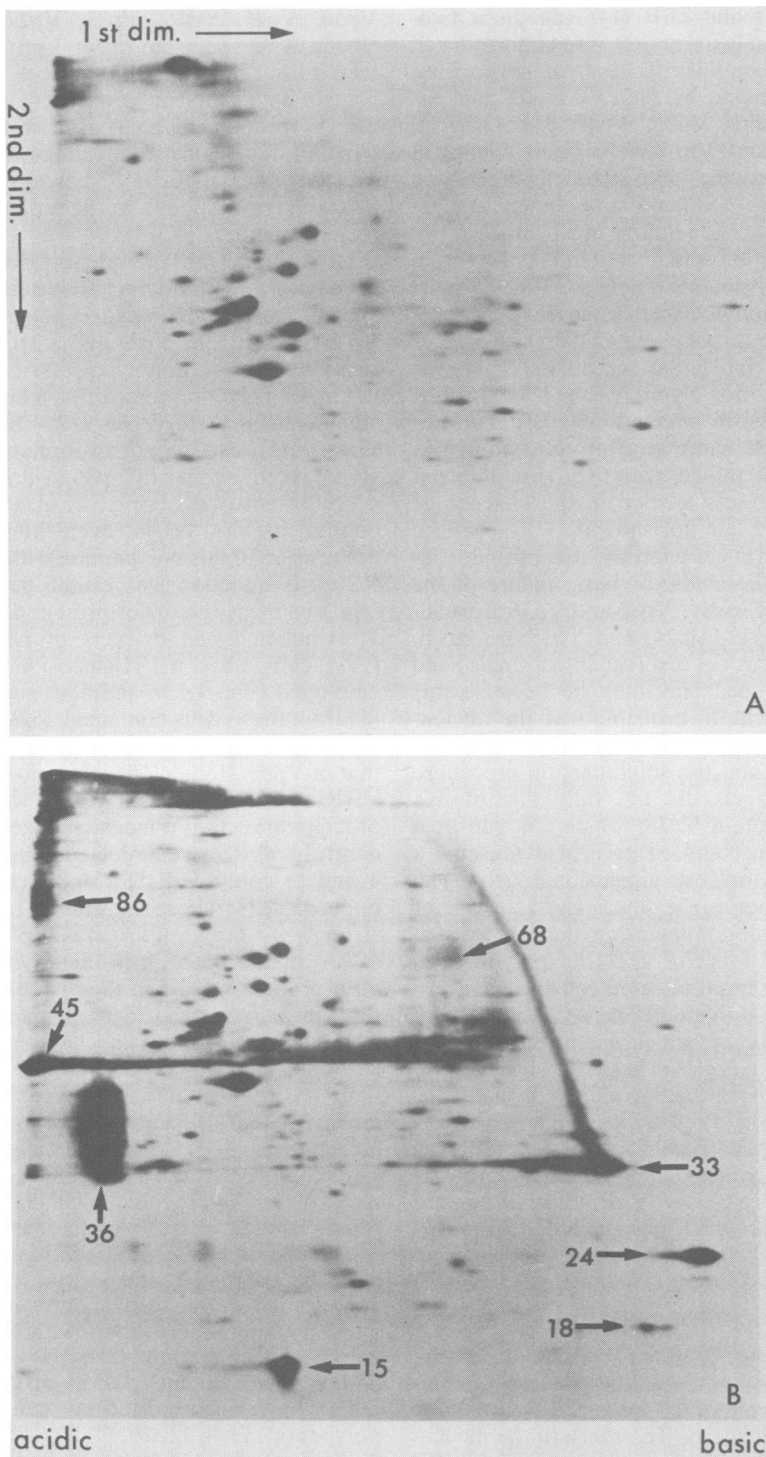


FIG. 4. Separation of control (A) and RS virus-infected (B) LLC-MK<sub>2</sub> cell proteins in two-dimensional gels by NEPHGE. RS virus-infected LLC-MK<sub>2</sub> cells were labeled for 3 h with [<sup>3</sup>H]leucine, beginning 24 h postinfection. Cells were solubilized with 0.5% NP-40 and processed as described in the text. Samples were electrophoresed on 13.5-cm-long gels at 500 V for 5 h. After equilibration in sample buffer, the gels were immobilized with 1% agarose on top of a 0.75-mm-thick 12.5% polyacrylamide gel. A sample of infected cell extract was included on the slab gel to provide markers for the RS virus proteins.

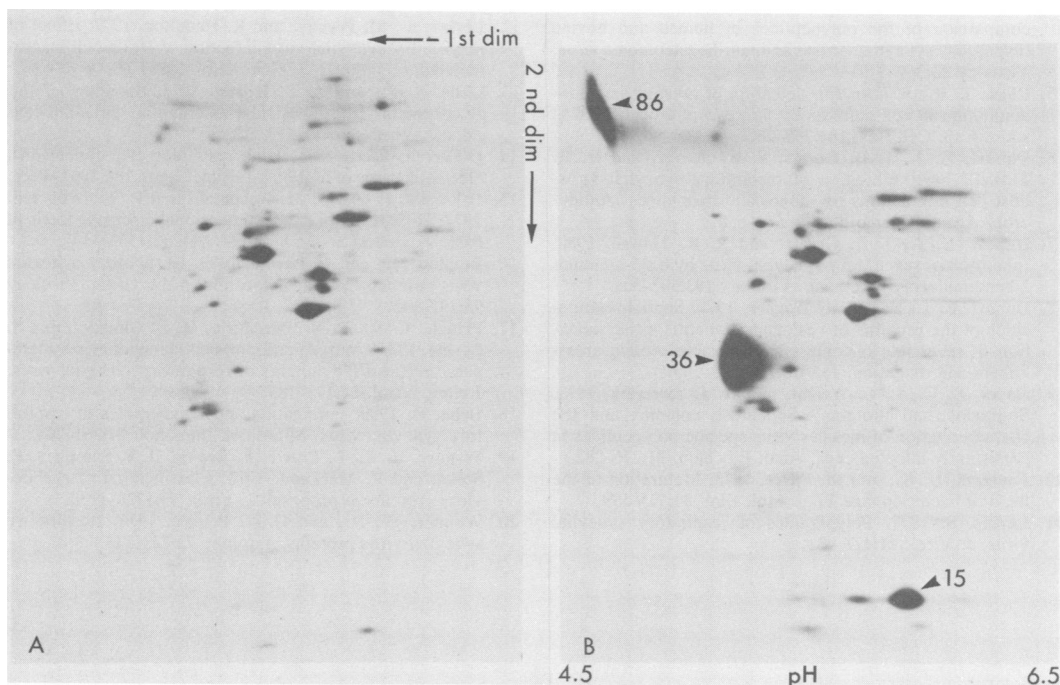


FIG. 5. IEF of control (A) and RS virus-infected (B) cell extracts. Cells were labeled and solubilized as described in the legend to Fig. 4. First-dimension gels were run for 18 h at 350 V, followed by 1 h at 700 V. The pH of the first-dimension gel was measured by suspending 1-cm segments of a parallel gel in 1 ml of water.

precursor to another RS virus protein(s). Cash et al. (3) detected a polypeptide of 61,000 daltons (VI61) in *in vitro* translation experiments with mRNA from RS virus-infected cells. Recent speculation on the nature of VI61 suggested that it might be the unglycosylated precursor for the glycoprotein GP1 (Vp86) (17). The data in Fig. 1 make this highly unlikely if VI61 and Vp68 are identical polypeptides, since Vp68 is labeled with [<sup>35</sup>S]methionine whereas Vp86 is not.

Two viral proteins of less than 20,000 daltons were demonstrated in this study. Two-dimensional PAGE analysis showed that these proteins are unique as Vp18 is basic, whereas Vp15 is acidic. These are believed to be primary gene products in that the inclusion of protease inhibitors did not alter the polypeptide profile found in Fig. 1 (data not shown). No previous report has identified both of these proteins. Cash et al. (4) reported the existence of a 10,500-dalton protein in RS virus-infected cells, and Bernstein and Hruska (1) detected a 17,000-dalton protein which appeared to label with [<sup>3</sup>H]glucosamine. Attempts to label Vp18 with [<sup>3</sup>H]glucosamine were not successful, but a diffuse band of [<sup>3</sup>H]glucosamine label appeared to be associated with Vp24 (data not shown).

At present, there are eight identified complementation groups of RS virus (17). This study

has presented evidence for at least nine viral proteins in RS virus-infected cells by using a combination of one-dimensional and two-dimensional PAGE. The function of each of these polypeptides has not been established. In particular, the role of Vp86 in the replication cycle needs to be established. Work is now in progress to determine whether the amidine inhibitors interact with Vp86.

#### ACKNOWLEDGMENTS

I thank Gail McKenzie for her excellent technical assistance.

The work was supported by Public Health Service grant HL-19171 awarded by the National Heart, Lung, and Blood Institute and by a University of North Carolina Medical Faculty grant.

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