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VIROLOGY 208, 121–131 (1995)

The Paramyxovirus SV5 V Protein Binds Two Atoms of Zinc and Is a Structural Component of Virions

REAY G. PATERSON,*[,1] GEORGE P. LESER,* MARGARET A. SHAUGHNESSY,† and ROBERT A. LAMB*†

*Department of Biochemistry, Molecular Biology and Cell Biology; and †Howard Hughes Medical Institute, Northwestern University, Evanston, Illinois 60208–3500

Received November 21, 1994; Accepted January 10, 1995

The paramyxovirus simian virus 5 (SV5) cysteine-rich V protein has been shown to be a virus structural protein by analysis of the polypeptides of purified SV5 virions. In addition, the V protein has been identified as a component of the virus nucleocapsid core both by the analysis of the polypeptides present in radioactively labeled preparations of purified nucleocapsids and by immunoelectron microscopy. Quantitative autoradiography was used to determine that there are ~350 molecules of the V protein in virions. The V protein has been purified from V recombinant baculovirus-infected insect cells and by using inductively coupled argon plasma atomic emission spectroscopy it was found that each molecule of V binds two zinc atoms. © 1995 Academic Press, Inc.

INTRODUCTION

Simian virus 5 (SV5) is a prototype paramyxovirus which has a single-stranded, negative-sense genomic RNA (vRNA) approximately 15,000 nucleotides in length that codes for eight polypeptides, the nucleoprotein (NP), phosphoprotein (P), V protein (V), matrix protein (M), fusion protein (F), small hydrophobic protein (SH), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L). The genomic RNA is present in the virus particle in a complex with the NP to form the nucleocapsid. Associated with the nucleocapsid are the P and L proteins which form the virus transcriptase complex. Surrounding the RNP complex is the virus envelope derived from the host-cell plasma membrane and underlying the envelope is the M protein. Inserted into the virus envelope are the major virus-encoded integral membrane proteins HN, which possesses both hemagglutinin and neuraminidase activities and functions to attach the virus to the target cell membrane via its receptor sialic acid, and F, which is responsible for introducing the nucleocapsid into the cytoplasm by mediating fusion of the virus envelope with the host-cell plasma membrane (reviewed in Lamb and Kolakofsky, 1995). At present it is not known whether the third SV5 encoded integral membrane protein, SH (Hiebert *et al.*, 1985), is a structural component of the virion.

The paramyxovirus V protein has been classified as a nonstructural protein as it was found to be synthesized in virus-infected cells but was not found in virions (Peluso *et al.*, 1977). The V protein is encoded by the same gene as the P protein by a mechanism that is unique to the P genes of paramyxoviruses. In SV5 the V protein is translated from a mRNA that is a faithful copy of the P gene, while the P protein is translated from a mRNA that contains two additional nucleotides that are not templated by the genome RNA (Thomas *et al.*, 1988). The mechanism by which this occurs has been termed pseudotemplated transcription (Jacques and Kolakofsky, 1991) and as its name suggests, appears to occur cotranscriptionally and is a function of the virus transcriptase (Vidal *et al.*, 1990). As a result of the nontemplated addition of two G residues to the P mRNA, translation initiates in the 0 reading frame and at the insertion point switches to the +1 reading frame. Thus, the P and V proteins share 164 N-terminal amino acids and have unique C termini (Thomas *et al.*, 1988). The C terminus of V is highly conserved among the V proteins of paramyxoviruses (Thomas *et al.*, 1988) and contains seven conserved cysteine residues in a motif that has homology to zinc finger domains which have been identified in many transcription factors and nucleic acid binding proteins (for review see O'Halloran, 1993). The homology to zinc finger domains led to the suggestion that the V protein would have the ability to bind zinc (Thomas *et al.*, 1988) and using a zinc blot assay this has been shown to be the case for the V protein of measles virus (Liston and Briedis, 1994).

In this paper we provide evidence that the SV5 V protein is a structural component of the SV5 virion and is associated with nucleocapsids. Furthermore analysis of purified V protein by using inductively coupled argon plasma atomic emission spectroscopy experiments indicates that the SV5 V protein binds zinc and that there are two zinc atoms per molecule of V protein.

[1] To whom correspondence and reprint requests should be addressed at Department of Biochemistry, Molecular Biology and Cell Biology, 2153 North Campus Drive, Northwestern University, Evanston, IL 60208–3500. Fax: (708) 491-2467. E-mail: r-paterson@nwu.edu.

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MATERIALS AND METHODS

Cells

Monolayer cultures of a variant of the MDBK line of bovine kidney cells, a variant of the MDCK line of canine kidney cells, and the TC7 clone of CV-1 cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum. Monolayer cultures of *Sf9* cells were grown in Grace's insect cell culture medium (supplemented) containing 10% fetal calf serum. Suspension cultures of *Sf9* cells in microcarrier spinner flasks (Bellco Glass, Inc., Vineland, NJ) were grown in Grace's cell culture medium (supplemented) containing 10% fetal calf serum and 0.1% Pluronic F-68 (Gibco BRL, Gaithersburg, MD).

Virus infections and radioactive labeling of cells

Stock virus was grown in MDBK cells infected with the W3 strain of SV5 (Choppin, 1964) as described previously (Peluso *et al.*, 1977). For biochemical experiments, CV-1 cells were used and infected as described previously (Paterson *et al.*, 1984; Thomas *et al.*, 1988). For growth of radioactive virus MDCK cells were infected and labeled from 3 to 5 days postinfection (p.i.) in 90% methionine- and cysteine-deficient DME and 10% complete DME with either a combination of Tran[^{35}S]label (ICN Radiochemicals, Irvine, CA) and [^{35}S]cysteine (Amersham Corp., Arlington Heights, IL), [150 μCi (30 μCi/ml) and 50 μCi (10 μCi/ml), respectively] or [^{35}S]cysteine alone [200 μCi (40μCi/ml)]. Recombinant baculoviruses (*Autographa californica* nuclear polyhedrosis virus) were grown in *Sf9* cells as described (O'Reilly *et al.*, 1992; Summers and Smith, 1987).

SV5 purification and nucleocapsid preparation

The supernatant medium from SV5-infected cells was clarified by centrifugation at 3000 rpm in an IEC DPR-6000 centrifuge using a 253 12-place rotor. Virus was pelleted by centrifuging at 18,000 rpm in a type 19 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 1 hr at 4°. The virus pellet was resuspended in NTE (100 m*M* NaCl, 10 m*M* Tris–HCl [pH 7.4], 1 mM EDTA) and layered on top of a linear 15–60% (w/v) sucrose/NTE gradient. The gradient was centrifuged (24,000 rpm at 4° for 1 h) in a Beckman SW41 Ti rotor and 15 0.75-ml fractions were collected from the bottom of the tube. A sample (0.2 ml) of each virus fraction from a gradient containing ^{35}S-labeled virus was trichloroacetic acid (TCA) precipitated and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described below. The fractions containing the virus were diluted in NTE and the virus pelleted by centrifuging at 40,000 rpm for 45 min at 4° in a Beckman type 70 Ti rotor. Finally, the virus was suspended in NTE and subjected to further analysis.

Purified ^{35}S-labeled virus was made 2% (v/v) Triton X-100 and 500 m*M* NaCl, incubated at 30° for 1 hr and nucleocapsids were prepared by two alternative methods. (1) D_2O sucrose gradient centrifugation: Solubilized virus was layered on top of a 15–65% (w/v in NTE made with D_2O) sucrose gradient and the gradient was centrifuged (58,000 rpm at 4°, 3.5 hr) in a Beckman SW60 Ti rotor (Kingsbury and Darlington, 1968). Fourteen 0.3-ml fractions were collected from the bottom of the tube, diluted by the addition of 0.2 ml NTE, and 0.25 ml analyzed by TCA precipitation and SDS–PAGE. (2) Cesium chloride (CsCl) gradient centrifugation: Solubilized virus was layered on top of a 25–40% (w/v in NTE) CsCl gradient containing 0.5 *M* urea and centrifuged at 39,000 rpm for 3 hr at 20° in a Beckman SW60 Ti rotor (Mountcastle *et al.*, 1970). The visible nucleocapsid band was collected, diluted in NTE and pelleted by centrifuging at 40,000 rpm for 25 min at 4° in a Beckman TLS 55 rotor, dissolved in sample buffer (4% [w/v] SDS, 40% [w/v] glycerol, 3% [w/v] dithiothreitol, 60 m*M* Tris–HCl [pH 6.8], a few grains of bromophenol blue) and analyzed by SDS-PAGE.

Construction of recombinant V-baculovirus.

The *A. californica* nuclear polyhedrosis virus (AcMNPV) transfer vector pVL941 (Luckow and Summers, 1989) was digested with *Bam*HI, filled in with T4 DNA polymerase, and *Xba*I linkers were added. The V cDNA was excised from pGEM-2 by digestion with *Xba*I and ligated into the adapted pVL941. The pVL941-V plasmid DNA and circular wild type AcMNPV DNA were introduced into *Sf9* cells by calcium phosphate coprecipitation and V recombinant baculovirus was isolated according to standard protocols (O'Reilly *et al.*, 1992)

Purification of recombinant baculovirus expressed V protein

Suspension cultures of *Sf9* cells at a density of 1 × 10^6 cells/ml were infected with the V recombinant baculovirus at a moi of 5 PFU/cell. At 72 hr p.i. the infected cells were harvested and suspended in 10 m*M* NaCl, 10 mM Tris-HCl pH 7.4, 1.5 m*M* $MgCl_2$ containing 0.1 mg/ml α2-macroglobulin, 0.1 m*M* PMSF, 2 μg/ml calpain inhibitor I, 0.1 m*M* 4-(2-aminoethyl) benzensulfonyl fluoride, 25 μg/ml antipain, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 25 μg/ml chymostatin, and 25 μg/ml pepstatin. The cell suspension was incubated on ice for 10 min and lysed by Dounce homogenization followed by passing through a 25G needle. The NaCl concentration was adjusted to 20 m*M* and the lysates were centrifuged at 40,000 rpm in a Beckman Type 70 Ti rotor for 30 min at 4° to pellet the cell debris.

The cleared lysate was loaded onto a Cibacron Blue 3GA (Sigma, St. Louis, MO) column equilibrated in 20 m*M* Tris–HCl, 20 m*M* NaCl, pH 7.4, and bound proteins were eluted using a 50 m*M* to 1 *M* NaCl gradient in 20 m*M* Tris–HCl pH 7.4. The fractions which contained significant amounts of the V protein were pooled and dialyzed against 20 m*M* Tris-HCl, 20 m*M* NaCl, pH 7.4.

The dialyzed proteins were then applied to a DEAE Sepharose CL-6B (Pharmacia Biotech Inc., Piscataway, NJ) column equilibriated in 20 m*M* Tris-HCl, 20 m*M* NaCl, pH 7.4, and the bound proteins were eluted using a NaCl step gradient consisting of 50 m*M* NaCl, 100 m*M* NaCl, and 1 *M* NaCl all in 20 m*M* Tris-HCl, pH 7.4. The peak V containing fractions were pooled, dialyzed against 50 m*M* 4-morpholineethanesulfonic acid (MES), 20 m*M* NaCl, pH 6.5, and loaded onto a prepacked HiTrap SP column (Pharmacia) equilibriated in 50 m*M* MES, pH 6.5. The V protein was eluted using a NaCl step gradient consisting of 50 m*M*, 100 m*M*, 200 m*M*, 300 m*M*, 400 m*M*, 500 m*M* and 1 *M* NaCl in 50 m*M* MES. The fractions collected from the HiTrap SP column were analyzed by SDS–PAGE and Coomassie brilliant blue staining and the V protein was estimated to be >95% pure.

Antibodies, immunoprecipitation, immunoblotting, and indirect immunofluorescence.

Aliquots (0.15 ml) of the gradient fractions containing the peak of [^{35}S]-labeled virus proteins (fractions 4–7) were added to an equal volume of 2× RIPA buffer (0.3 *M* NaCl, 2% [w/v] sodium deoxycholate, 2% [v/v] Triton X-100, 0.2% [w/v] SDS, 0.2 *M* Tris–HCl [pH 7.4], 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 420 ng/ml aprotinin, 20 m*M* iodoacetimide) and immunoprecipitated with either monoclonal antibody (mAb) NPa or mAb Pe (specific for the SV5 NP and P proteins, respectively) or a rabbit polyclonal antibody (αV) raised against a synthetic peptide specific for V residues 168–183. Immunoprecipitation was performed as described previously (Lamb *et al.*, 1978) and the immunoprecipitated proteins analyzed by SDS–PAGE as described below.

For immunoblotting, virus polypeptides from gradient fractions 4–7 (60 μl) were separated by SDS–PAGE and transferred to nitrocellulose and immunoblotting was performed essentially as described previously (Burnette, 1981; Paterson and Lamb, 1993). The primary antibodies used were mAb NPa, mAb Pk (specific for the SV5 P and V proteins), and a rabbit polyclonal antibody (αL) raised against an L protein-specific synthetic peptide (residues 2241–2254) (Parks, 1994). The secondary antibodies used were peroxidase-conjugated goat anti-mouse IgG (F (ab')$_2$ fragment specific) and peroxidase conjugated goat anti-rabbit IgG (F (ab')$_2$ fragment specific) (Cooper Biomedical Inc., Malvern, PA). The immobilized proteins were detected by light-enhanced chemiluminescence according to the manufacturer's instructions (ECL, Amersham Corp., Arlington Heights, IL).

The V protein-specific monoclonal antibodies (mAbs V-11, V-14, V-26) were prepared using V protein purified from recombinant baculovirus-infected cells as the antigen by standard methods (Harlow and Lane, 1988). The final screening of the specificity of the mAbs was by immunoprecipitation to identify antibodies that were specific for V and had no reactivity for the P protein. The SV5 NP (NPa) and P (Pe and Pk) specific monoclonal antibodies (Randall *et al.*, 1987) were kindly provided by Dr. Rick E. Randall (St. Andrews University, St. Andrews, UK).

Indirect immunofluorescence was carried out as described previously (Paterson and Lamb, 1993; Paterson *et al.*, 1989). Coverslips of infected CV-1 cells were prepared for fluorescence by fixing for 5 min at room temperature in 0.5% solution of methanol-free formaldehyde (Polysciences Inc., Warrington, PA) followed by permeabilization using acetone at −20°C for 2 min. Ascites fluid to mAb Pe was diluted 1:100 in 1% BSA/PBS and tissue culture fluid containing mAbs V-11, V-14, and V-26 was diluted 1:5 in 1% BSA/PBS. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch, West Grove, PA) was used as the secondary antibody.

Electron microscopy and immunocytochemistry

Colloidal gold and rabbit anti-mouse IgG coupled to 10-nm gold particles were prepared as described (Slot and Geuze, 1985). Intact SV5 virions were prepared for staining by diluting virus purified on a sucrose gradient 1:10 in 100 m*M* NaCl, 10 m*M* Tris, pH 7.4, 1 m*M* EDTA (NTE). A drop of diluted virus was placed on a parlodion covered nickel grid and allowed to absorb onto it for 30 sec. Disrupted SV5 virus was similarly prepared for staining except purified virus was diluted 1:10 in lysis buffer (0.5% Na deoxycholate, 1% NP-40, 50 m*M* Tris, pH 8, 0.15 *M* NaCl). Purified SV5 nucleocapsids were prepared on CsCl gradients as described above. Nucleocapsids were diluted 1:20 in NTE and allowed to absorb onto parlodion-coated nickel grids for 30 sec–2 min. All preparations were stained using a protocol adapted from Murti *et al.*, (1985). Following absorption onto grids, samples were washed by floating on a drop of Tris-buffered saline, pH 7.4 (TBS), for 5 min, and then blocked by incubation on drops of 3% ovalbumin in TBS for 45 min. Grids were washed with TBS for 5 min and then reacted for 1 hr with either mAb V-11 tissue culture supernatant (undiluted) or ascites fluid containing antibodies specific for either the influenza A virus M_2 protein (14C2) (Zebedee and Lamb, 1988) or SV5 NP polypeptide (Randall *et al.*, 1987), both diluted 1:300 in 1% ovalbumin in TBS. Following three successive washes on drops of TBS for 10 min each, samples were incubated for 1 hr with goat anti-mouse IgG coupled to 10-nm gold particles diluted 1:10 in 1% ovalbumin in TBS. After washing with TBS as before, grids were negatively stained with 2% phosphotungstic acid, pH 6.6. Prior to viewing, a thin layer of carbon was evaporated onto the grids. All incubations were carried out in a humidified chamber at room temperature and all solutions were filtered before use. Following absorption of the virus, grids were not allowed to dry until after they were negatively stained.

For the competition experiment disrupted SV5 virions

were stained with 3.5-fold concentrated mAb V-11 that had been preincubated with purified V protein. Grids were then reacted with either concentrated V antibody diluted 1:3 in 1% ovalbumin in TBS as a control or diluted 1:3 in purified V protein (247 μg/ml) in 50 m*M* MES, 100 m*M* NaCl that had been adjusted to approximately pH 7.4 by the addition of 1 *M* Tris, pH 8. Diluted V antibody was allowed to preincubate at room temperature for 45 min prior to its use in staining grids.

Polyacrylamide gel electrophoresis, autoradiography, and fluorography.

Polypeptides were analyzed by SDS–PAGE on 15% Tris–glycine gels as described previously (Lamb *et al.*, 1978). Gels were processed for fluorography as described previously (Lamb and Choppin, 1976) or were analyzed by quantitative radiography using a phosphorimager (PhosphorImager System 400, Molecular Dynamics, Sunnyvale, CA). Gels were stained with Coomassie brilliant blue to visualize recombinant baculovirus expressed V protein.

Quantification of the zinc content of the purified SV5 V protein.

An Atom Scan 25 atomic emission spectrophotometer (Thermal Jarrell Ash Corp., Franklin, MA) employing inductively coupled argon plasma atomic emission spectra (ICAP-AES) to measure the zinc emission spectrum (wavelength = 213.836 nm) was used to quantify the amount of zinc bound by the purified recombinant V protein. A zinc standard solution suitable for ICAP-AES (Aldrich, St. Louis, MO) was diluted to 2 ppm in 50 m*M* MES, 50 m*M* NaCl, pH 6.5, and the Atom Scan 25 was standardized using the 2 ppm zinc standard and the buffer alone as a blank. Carbonic anhydrase (Sigma) was dissolved in 50 m*M* MES, 50 m*M* NaCl, pH 6.5, and samples containing five different concentrations of the protein were scanned for zinc content as a positive control for the ICAP-AES.

The concentration of the carbonic anhydrase and V protein in the scanned samples was determined by the BCA protein assay (Pierce, Rockford, IL) using the enhanced protocol as described by the manufacturers. A standard curve was prepared using carbonic anhydrase and the V protein concentration was determined by interpolating off the standard curve.

RESULTS

Analysis of the protein composition of SV5 virus particles

Since the time of the early experiments that characterized the SV5 V protein as a nonstructural protein (Peluso *et al.*, 1977) we have obtained the nucleotide sequence of a cDNA encoding the V protein and thus the predicted amino acid sequence (Thomas *et al.*, 1988). With the

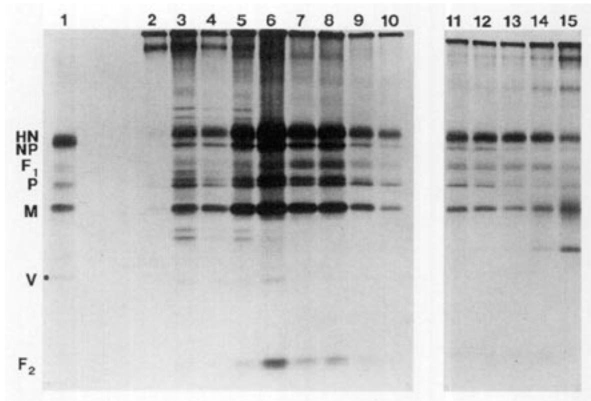


FIG. 1. Polypeptide composition of purified SV5 virus. SV5 virus was grown and labeled with Tran[^{35}S]label and [^{35}S]cysteine and subjected to sedimentation on a 15–60% sucrose gradient. Fractions were collected from the bottom of the gradient and analyzed by TCA precipitation and SDS–PAGE. A fluorograph of a 15% polyacrylamide gel is shown. Lane 1, SV5-infected cell lysate labeled with Tran[^{35}S]label. Lanes 2–15; gradient fractions 1–14. The bottom of the gradient is at the left. The V protein is indicated by the filled circle on the left of the figure.

knowledge that the V protein is rich in cysteine residues we decided to reexamine the question of whether V is a component of purified SV5 virus. To increase our chances of detecting the V protein, SV5 virus was labeled with a mixture of Tran[^{35}S]label and [^{35}S]cysteine and grown and purified as described under Materials and Methods. A 15–60% sucrose gradient was used to purify the virus and the results of SDS–PAGE analysis of proteins that were TCA precipitated from gradient fractions are shown in Fig. 1. The peak of viral polypeptides was detected in lanes 5–8 (corresponding to fractions 4–7 from the bottom of the gradient) and these fractions were also found to contain a polypeptide that had an electrophoretic mobility consistent with that observed for the V protein. Gradient fractions 4–7 were therefore used for further analysis of virus proteins. A second peak containing the HN, F, and *M* proteins was detected in fractions near the top of the gradient (lanes 11–14) and this is thought to be due to the presence of plasma membrane vesicles as these fractions are virtually devoid of the NP and P proteins.

As the putative V protein was detected in relatively small amounts when the SV5 virus proteins were analyzed directly by SDS–PAGE we sought immunological evidence that the V protein was a component of purified virions by using an anti-V specific peptide sera. ^{35}S-labeled virions contained in gradient fractions 4–7 were detergent disrupted and proteins immunoprecipitated using either monoclonal antibodies specific for the NP (Fig. 2A lanes 2–5) and P (Fig. 2A lanes 10–13) proteins or a rabbit polyclonal antibody specific for the V protein (Fig. 2A lanes 6–9). The V protein was clearly detected in Fig. 2A, lanes 6–9, thus supporting the conclusion drawn from direct SDS–PAGE analysis of virion proteins that the V protein is present in SV5 virions. Note in Fig.

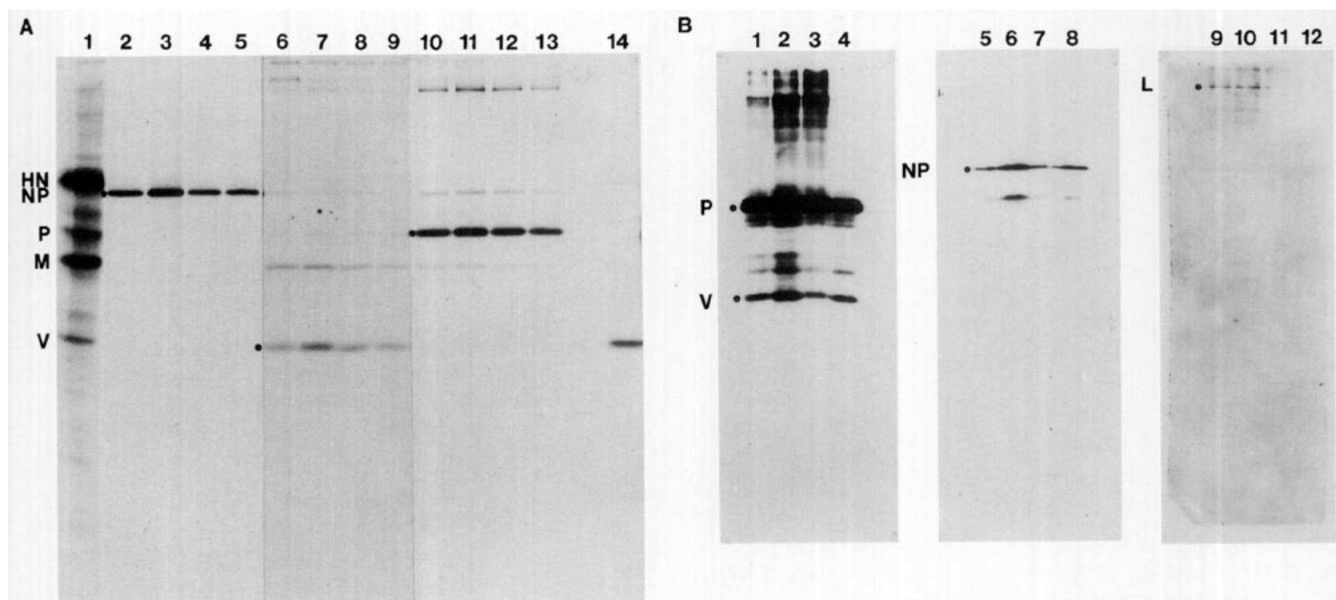


FIG. 2. Immunoprecipitation and immunoblotting of virions from sucrose gradient fractions. (A) Fractions containing the peak of ^{35}S-labeled virus proteins (Fig. 1, lanes 5–8) were immunoprecipitated and analyzed by SDS–PAGE and fluorography. Lanes 2–5, α-NP mAb; lanes 6–9, α-V peptide sera; lanes 10–13, α-P mAb. Lane 1, SV5-infected cell lysate labeled with Tran[^{35}S]label; lane 14, V protein immunoprecipitated from SV5-infected cells. The V protein is indicated by the filled circle. Lanes 6–9 were exposed for longer than the rest of the gel. (B) Unlabeled virus proteins from sucrose gradient fractions 4–7 (Fig. 1) were separated by SDS–PAGE, transferred to nitrocellulose, and probed with mAb Pk (specific for the P and V proteins) (lanes 1–4), mAb NPa (lanes 5–8), and a rabbit polyclonal sera specific for the L protein (lanes 9–12). The protein-antibody complexes were visualized using light-enhanced chemiluminescence. The V, P, NP, and L proteins are indicated.

2A lanes 10–13 that the P antibody appears to coprecipitate L protein as described previously (Parks, 1994).

Another means of identifying the V protein as a specific component of virions was to perform an immunoblot on virus polypeptides separated by SDS–PAGE from gradient fractions 4–7. Polypeptides were transferred to nitrocellulose and the immunoblot carried out using a mAb specific for both P and V (Pk), the NP-specific monoclonal antibody, or a rabbit polyclonal antibody specific for the SV5 L protein as the primary antibodies. The immobilized proteins were detected by light-enhanced chemiluminescence as described under Materials and Methods. As shown in Fig. 2B the V and P proteins (lanes 1–4) were detected in gradient fractions that also contained NP (lanes 5–8) and the L protein (lanes 9–12).

Nucleocapsid preparation.

To investigate the subviral location of the V protein nucleocapsids were prepared by treating purified ^{35}S-labeled virus with Triton X-100 and 0.5 *M* NaCl and layering the solubilized virus on top of 15–65% sucrose gradients containing D_2O as described under Materials and Methods. Fractions were collected from the bottom of the gradient and analyzed by TCA precipitation and SDS–PAGE (Fig. 3). The nucleocapsids banded in fractions 5 and 6 and contained the NP, P, and V polypeptides and some *M* polypeptide. However, the majority of the *M* protein was stripped from the nucleocapsids by treatment of the virus with Triton X-100 and 0.5 *M* NaCl and was found toward the top of the gradient in fractions containing the F and HN glycoproteins (Fig. 3, lanes 9–14). The panel on the right in Fig. 3 shows a longer autoradiographic exposure of fractions 5 and 6 in which the V protein is more readily detected.

Another method of preparing nucleocapsids, which is often considered even more stringent, is to subject the solubilized virus to cesium chloride gradient centrifugation. This was done and the visible nucleocapsid band

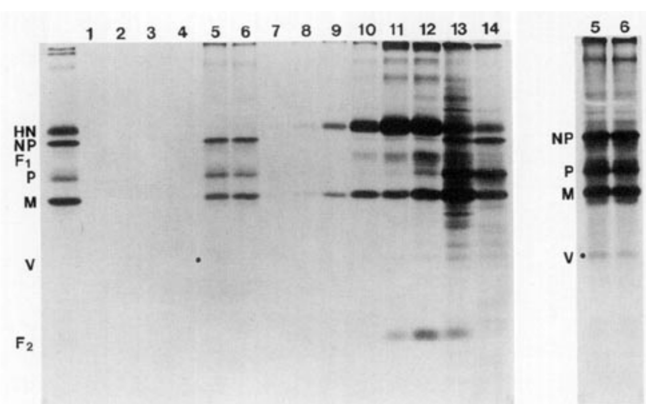


FIG. 3. Preparation of nucleocapsids by sedimentation in sucrose gradients containing D_2O. Purified ^{35}S-labeled virus was treated with 2% Triton X-100 and 0.5 *M* NaCl, layered onto 15–65% sucrose gradients, and centrifuged as described under Materials and Methods. Gradient fractions were analyzed by TCA precipitation and SDS–PAGE. Lanes 1–14 are gradient fractions 1–14 with fraction 1 being the bottom of the gradient. The lane at the immediate left of the figure is purified SV5 virus. The panel at the right is a longer exposure of lanes 5 and 6. The V protein is indicated by the filled circle.

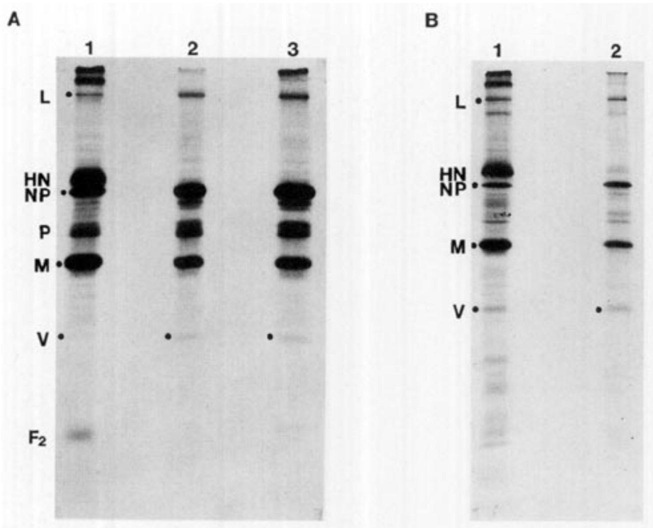


FIG. 4. Preparation of nucleocapsids by sedimentation in CsCl gradients. Purified ^{35}S-labeled virus was treated with 2% Triton X-100 and 0.5 *M* NaCl, layered on top of a 25–40% CsCl gradient, and centrifuged as described under Materials and Methods. The visible nucleocapsid band was collected, pelleted, dissolved in sample buffer, and analyzed by SDS–PAGE. (A) Nucleocapsids labeled with Tran[^{35}S]label and [^{35}S]cysteine. Lane 1, purified SV5 virus. Lanes 2 and 3, two different nucleocapsid preparations derived from the virus shown in lane 1. (B) Nucleocapsids labeled with [^{35}S]cysteine. Lane 1, purified SV5 virus. Lane 2, nucleocapsids prepared from the virus sample shown in lane 1.

was collected, and the nucleocapsids were pelleted and analyzed by SDS–PAGE. Two different nucleocapsid preparations were analyzed (Fig. 4A, lanes 2 and 3) and it can be seen that some *M* protein remained associated with the nucleocapsids although in small amounts relative to the *M* protein that is present in virus (Fig. 4A, lane 1). The V protein was seen in both nucleocapsid preparations (Fig. 4A, lanes 2 and 3) and the ratio of V to M in the nucleocapsids increased compared to the ratio in virus (compare lane 1 with lanes 2 and 3). Thus, these data indicate that the majority of the V protein fractionated with the nucleocapsids. However, from these data it cannot be distinguished whether the V protein associates directly with the RNP (RNA–NP–P–L complex) or whether the protein associates with a subclass of the M protein which in turn associates with the RNPs.

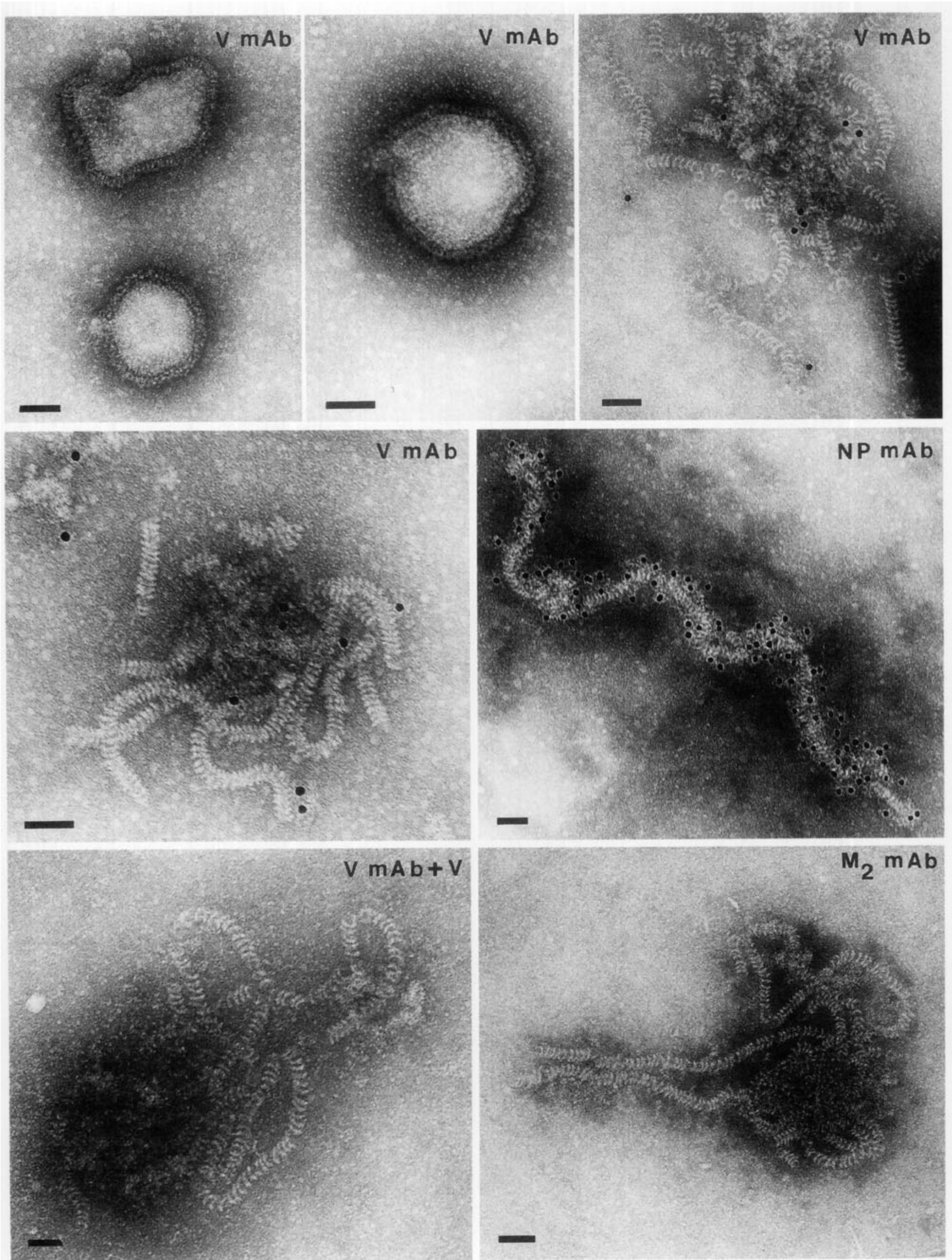
Quantification of the amount of V present in virus and nucleocapsids.

To quantify the amount of V protein in virus and nucleocapsids, virions were metabolically labeled with [^{35}S]cysteine, purified, and nucleocapsids prepared by cesium chloride centrifugation of detergent solubilized virus. The polypeptides of [^{35}S]cysteine-labeled virus and nucleocapsids are shown in Fig. 4B, lanes 1 and 2, respectively. The amount of radioactivity associated with the NP, M, and V bands in two gels such as the one shown in Fig. 4B was measured by quantitative radiography using a Phosphorimager. To quantify the amount of P protein in virus and nucleocapsids the same procedure was used except that virions were labeled with [^{35}S]methionine but in this case V cannot be readily detected. The radioactivity (Phosphorimager units) associated with V, P, and M was compared to that of NP and normalized using the known number of cysteine residues in each protein (V = 7; M = 9; NP = 5) or the known number of methionine residues present in each protein (M = 11, NP = 21, P = 8; in each case including the initiator methionine) (Parks *et al.*, 1992; Sheshberadaran and Lamb, 1990; Thomas *et al.*, 1988). From electron microscopic examination of Sendai virus nucleocapsids (Egelman *et al.*, 1989) and from experiments to determine the requirements for efficient replication of Sendai virus defective-interfering RNA (Calain and Roux, 1993) it has been concluded that each NP protein in the nucleocapsid contacts six nucleotides of RNA. In the case of SV5 which has a genome 15,246 nucleotides in length this would mean that there are ~2540 molecules of the NP protein in the virion. Thus, it could be calculated that in SV5 virions there are 348 molecules of V, 410 molecules of P, and 10,414 molecules of M, whereas in nucleocapsids there are 314 molecules of V, 198 molecules of P and 2144 molecules of the M protein. Seemingly, the V protein present in the RNP preparations is less susceptible to stripping by CsCl than P protein.

Immunoelectronmicroscopy of SV5 virions and nucleocapsids.

Another method of examining for the presence of a protein in virions, which is independent of concerns regarding the relative purity of an enveloped virus from contaminating membrane vesicles, is to localize the protein by using immunogold electron microscopy. To localize the V protein by immunogold electron microscopy a panel of V-specific monoclonal antibodies was raised using purified recombinant V protein as antigen. Purified SV5 virions and nucleocapsids were prepared and processed for electron microscopy as described under Materials and Methods. As shown in Fig. 5 (top left and center) when intact SV5 virions were incubated with a monoclonal antibody specific for V (mAb V-11) no immunogold staining was observed; however, when lysed virus (Fig. 5, top right) or purified nucleocapsids (Fig. 5,

FIG. 5. Immunoelectron microscopy of SV5 virions and nucleocapsids. Purified SV5 virus and nucleocapsids were prepared for immunogold labeling as described under Materials and Methods. (Top left and center) Intact SV5 virions reacted with mAb V-11; (top right) lysed SV5 virions reacted with mAb V-11. (Center left) SV5 nucleocapsids reacted with mAb V-11; (center right) SV5 nucleocapsids reacted with mAb NPa. (Bottom left) Lysed SV5 virions reacted with mAb V-11 in the presence of purified recombinant V protein; (bottom right) lysed SV5 virions reacted with an influenza M_2 mAb (14C2). The bars represent 50 nm.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V mAb
V mAb
NP mAb
V mAb+V
M2 mAb

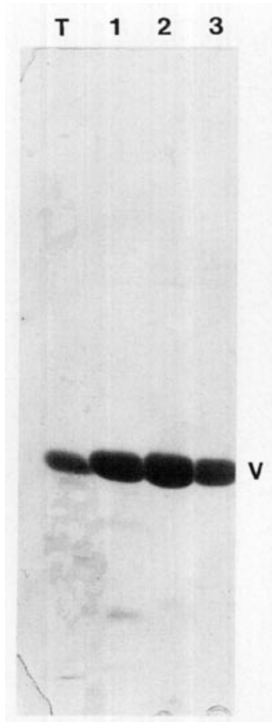

FIG. 6. Purification of recombinant SV5 V protein. The V protein was purified from *Sf9* cells infected with the V recombinant baculovirus as described under Materials and Methods. The fractions collected from a HiTrap SP column were analyzed by SDS–PAGE and the gel was stained with Coomassie brilliant blue. *T* = 40 μg trypsinogen marker. Lanes 1 – 3 fractions eluted from the column using 100 m*M* NaCl.

middle left) were incubated with mAb V-11 significant immunogold labeling was detected, although the density of gold particles was significantly lower than that observed when nucleocapsids were reacted with mAb NPa (Fig. 5, middle right). To show that the labeling of nucleocapsids with mAb V-11 was specific for the V protein lysed SV5 virions were incubated with mAb V-11 that had been preincubated with purified V protein. As shown in Fig. 5 (bottom left) this resulted in complete inhibition of immunogold staining. Another control for the specificity of the V mAb reactivity with nucleocapsids was to incubate nucleocapsids with an irrelevant mAb (14C2 specific for the influenza A virus M_2 protein) and as can be observed (Fig. 5, bottom right) no immunogold staining of SV5 nucleocapsids was observed. Thus, these data provide additional evidence for the V protein being a SV5 structural protein and also confirm the biochemical data discussed above that show that the V protein is a component of the nucleocapsid of purified SV5 virions. As discussed above, the nucleocapsid preparations contain M protein and it is not known whether the V protein associates directly with the RNP complex or whether the V protein associates with a subclass of the M protein which in turn associates with the RNPs.

The SV5 V protein binds two zinc atoms per molecule of V protein.

It has been shown for the measles virus V protein, by using a nonquantitative zinc-blotting technique, that the V protein binds zinc and this binding is determined by the cysteine-rich V-unique C-terminal domain: the P and V common N-terminal 164 residues do not bind zinc (Liston and Briedis, 1994). To determine quantitatively the number of moles of zinc per mole of the SV5 V protein, by using ICAP-AES, large quantities of purified SV5 V protein were required. Thus, a recombinant baculovirus that expressed the V protein was constructed and used to infect *Sf9* cells. The V protein was purified from the infected cells as described under Materials and Methods. Following chromatography on a Cibacron Blue 3GA, DEAE Sepharose CL-6B and finally a HiTrap SP column, the V protein was judged to be >95% pure as determined by SDS–PAGE and Coomassie blue staining of the HiTrap SP column fractions. The purified recombinant V protein (Fig. 6) was used to determine the zinc-binding capacity of V using the technique of ICAP-AES as described under Materials and Methods. As shown in Table 1, from the average values from many zinc emission measurements it was determined that one molecule of the SV5 V protein has the capacity to bind two zinc atoms.

Intracellular distribution of the SV5 V protein

The intracellular distribution of the V proteins of measles virus and Sendai virus had previously been examined by indirect immunofluorescence using antibodies raised against V-specific peptides (Curran *et al.*, 1991b; Wardrop and Briedis, 1991). The measles virus and Sendai virus V proteins were found to be evenly distributed throughout the cytoplasm of infected cells as shown by the diffuse cytoplasmic immunofluorescent staining pattern. We were therefore interested to compare the intracellular distribution of the SV5 V protein with those of measles virus and Sendai virus. The SV5 V monoclonal antibodies raised against purified recombinant protein were used to examine the intracellular distribution of V by indirect immunofluorescence of acetone-permeabilized SV5-infected CV-1 cells as described under Materials and Methods. The intracellular staining pattern of V obtained using three V-specific hybridoma secreted antibodies (Figs. 7C, 7D and 7E), is shown in comparison to the distribution of the SV5 P protein (Fig. 7A). At the present time it is not known whether the V-specific mAbs recognize the same or different epitopes. In addition, double-labeled fluorescence of P and V could not be

TABLE 1

Zinc/protein Ratios as Determined by ICP-AES

Protein	Experiment	Zinc/protein ratio
Carbonic anhydrase	1	0.918
Carbonic anhydrase	2	1.006
V	1	1.960
V	2	1.949
V	3	2.064

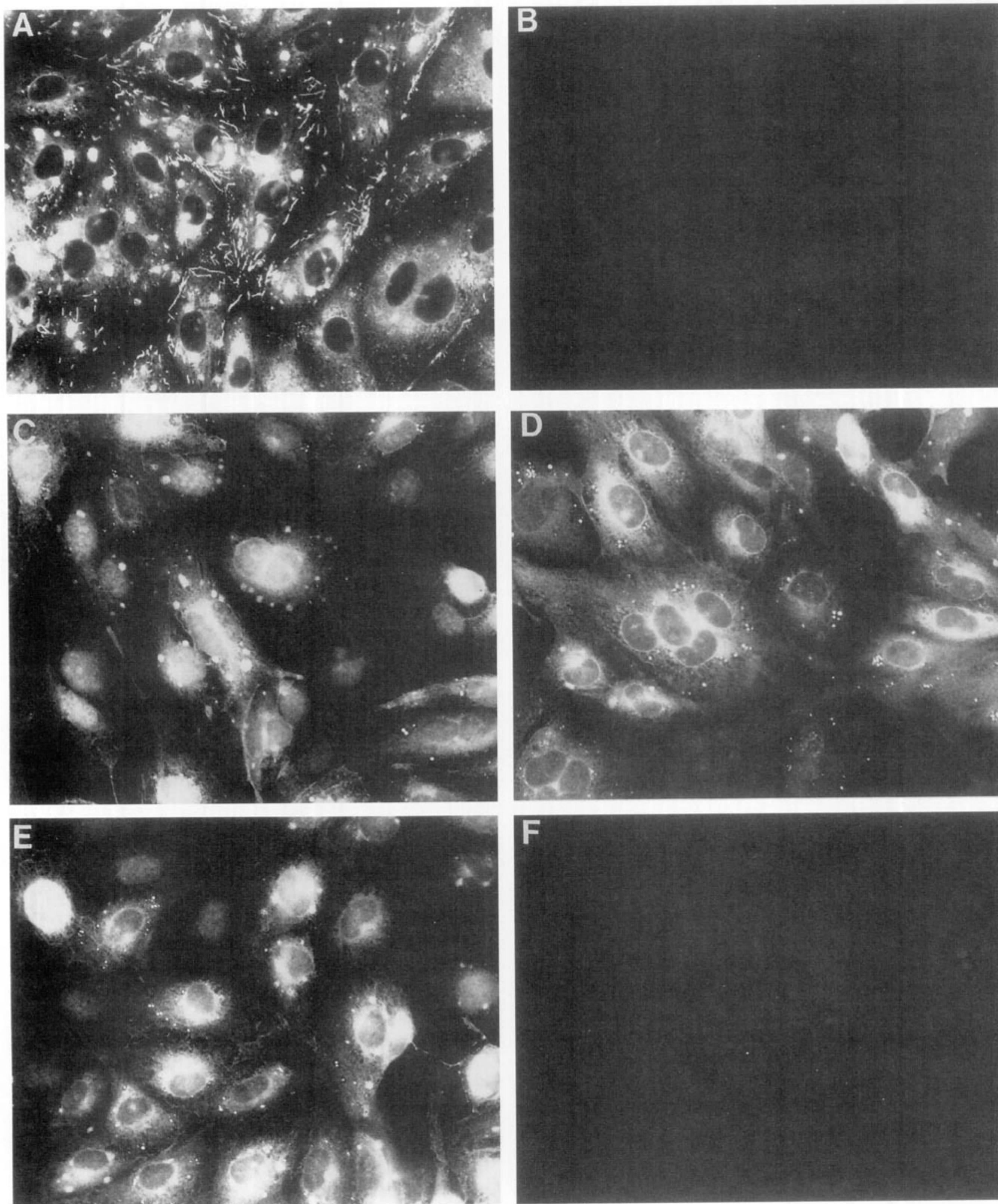

FIG. 7. Intracellular distribution of the SV5 V protein. SV5-infected and mock-infected CV-1 cells were prepared for indirect immunofluorescence staining with P- or V-specific mAbs and fluorescein isothiocyanate-conjugated secondary antibody as described under Materials and Methods. Panels (A, C, D and E) SV5-infected cells. (B and F) Mock-infected cells. Staining was done with the following antibodies: (A) mAb Pe, (B) mAb Pe, (C) mAb V-11, (D) mAb V-26, (E) mAb V-14, (F) mAb V-14. The photographic exposure for B and F was manually adjusted to be the same as for A, C, D, and E.

performed because of the inavailablity of primary antibodies of different isotypes. The intracellular distribution of P and V appeared to be subtly different (excluding the filamentous stained structures seen with the P mAb). Both P and V were detected throughout the cytoplasm as shown by the diffuse cytoplasmic immunofluorescence; however, the cytoplasmic aggregates that were detected using the P mAb were larger and more numerous than those detected using the V mAbs (compare Fig. 7A with Figs. 7C, 7D and 7E). In addition, diffuse nuclear staining was observed using the V mAbs (Figs. 7C, 7D, and 7E) whereas the P mAb did not stain the nuclei of infected cells (Fig. 7A). The filamentous structures stained with the P mAb and observed in Fig. 7 are out of the plane of focus of the cytoplasm. A very similar staining pattern has also been observed when using SV5 αHN sera (data not shown) and these filamentous structures are thought to be microvilli from which virions are budding.

DISCUSSION

The SV5 V protein was originally identified as a nonstructural protein that was present in SV5-infected cells (Peluso *et al.*, 1977). Now with the availability of V-specific antibodies and a knowledge of the predicted amino acid sequence of the V protein we readdressed the question of whether the V protein is a component of the SV5 virus particle. At the C-terminus of paramyxovirus V proteins there are seven conserved cysteine residues (Thomas *et al.*, 1988) arranged in a motif that resembles a zinc finger domain (for review see O'Halloran, 1993). The SV5 V protein contains only one methionine residue other than the initiation methionine residue. Thus, it was anticipated that by growing virus in the presence of [^{35}S]cysteine plus Tran[^{35}S]label the chances of detecting V in purified virus would be increased. This was found to be the case and the V protein was identified as a component of purified virus when examined by direct SDS–PAGE analysis of virus proteins (Fig. 1) and when examined by immunoprecipitation and immunoblotting using V-specific antibodies (Fig. 2). In addition, when nucleocapsids were separated from the other virus components the V protein was found to fractionate with the nucleocapsids (Figs. 3 and 4). Further evidence that the V protein is a component of SV5 virions and nucleocapsids was obtained by immunoelectron microscopy using a V-specific mAb (Fig. 5). Once the SV5 V protein was identified as a structural component of the virus particle it was important to determine how many molecules of the V protein were present in both virus particles and nucleocapsids. The approach that we used to do this was to label virus exclusively with [^{35}S]cysteine, prepare nucleocapsids, and carry out quantitative autoradiography on both virus and nucleocapsid proteins that had been separated by SDS–PAGE. From this approach it was calculated that there are ~350 molecules of V per virion or ~315 molecules of V per nucleocapsid. Taken together, all these data indicate that the V protein is a bonafide component of the nucleocapsid core and not just an adventitious contaminant. Nonetheless, as our nucleocapsid preparations contain about 20% of the total amount of the virion M protein, even after CsCl gradient centrifugation, it is not known whether the V protein is directly in contact with the RNP complex or whether the V protein associates with a specific subclass of M protein which in turn is associated with the RNP.

The intracellular locations of the P and V proteins, as examined by indirect immunofluorescence, indicate that the P and V proteins are distributed throughout the cytoplasm of SV5-infected cells and are also detected in cytoplasmic inclusions, although P protein-specific inclusions are more frequently observed than V protein-specific inclusions. The immunofluorescent staining pattern of the SV5 P protein is similar to the pattern of numerous brightly staining cytoplasmic inclusions found for the measles virus P protein (Wardrop and Briedis, 1991), whereas the Sendai virus P protein exhibits mostly a diffuse cytoplasmic distribution (Curran *et al.*, 1991b). The immunofluorescent staining pattern of the SV5 V protein is somewhat different from that reported for measles virus and Sendai virus as cytoplasmic inclusions were only observed for the SV5 V protein and not for the V proteins of either measles or Sendai virus. This difference may reflect the different types of antibodies used (mAb to native protein for SV5 versus V peptide-specific sera for measles virus and Sendai virus). The cytoplasmic P protein and V protein-specific inclusions may reflect areas of transcribing and replicating nucleocapsids and it will be of interest to compare the intracellular distribution of the SV5 NP, P, and V proteins by immunoelectron microscopy of thin sections of infected cells.

As discussed above, the paramyxovirus V proteins have a cysteine-rich domain at their C- termini reminiscent of a zinc binding domain and for the measles virus V protein it has been shown recently by using a nonquantitative blotting procedure that the cysteine-rich V-unique C-terminal domain binds zinc (Liston and Briedis, 1994). The quantitative ICAP-AES procedure described here to measure the amount of zinc in a sample of the SV5 V protein purified from V recombinant baculovirus-infected *Sf9* cells indicates that each molecule of V binds two zinc atoms. To understand the residues involved in coordinating zinc in the V-unique C-terminal domain mutations to change the conserved cysteine residues to alanine were introduced into the SV5 V protein and the altered molecules were expressed by using recombinant baculoviruses (unpublished observations). However, the cysteine-altered V proteins cannot be purified using the same scheme as described here for the wild-type V protein: thus, these single-residue changes may have caused a profound change in the folding of the V protein.

The function of V and its role in the paramyxovirus life cycle is not fully understood. When the effect of plasmid expressed Sendai virus V protein on the replication of a

Sendai virus defective-interfering RNA was examined (both *in vitro* and *in vivo)* the data suggested that the V protein has an inhibitory effect on genome replication (Curran *et al.*, 1991a, 1994). At the same time, however, it was found that the V protein had a slightly stimulatory effect on Sendai virus *in vitro* transcription (Curran *et al.*, 1994). Therefore, the exact role of V in virus RNA transcription and replication still remains to be determined. In addition, it is still not known whether the binding of zinc by the COOH terminus of V is necessary for the biological activity of the V protein.

ACKNOWLEDGEMENTS

We thank Ted Lin for excellent technical assistance. We are very grateful to Tom O'Halloran and Lisa Utschig-Johnson for advice with the ICP-AES analysis and the Northwestern University Chemistry Analytical Services Laboratory for access to the ICP-AES equipment. We are very grateful to Lynn Doglio for her expertise in monoclonal antibody production and for supervising the BMBCB, Northwestern University monoclonal antibody facility. This research was supported in part by Research Grant AI-23173 from the National Institute of Allergy and Infectious Disease. R.A.L. is an Investigator of the Howard Hughes Medical Institute.

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