



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Intracellular Accumulation of Punta Toro Virus Glycoproteins Expressed from Cloned cDNA

YUMIKO MATSUOKA,* TAKESHI IHARA,† DAVID H. L. BISHOP,† AND RICHARD W. COMPANS*¹

*Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294,
and †NERC Institute of Virology, Oxford, England, OX1 3SR, United Kingdom

Received April 4, 1988; accepted August 1, 1988

The Punta Toro virus (PTV) middle size (M) RNA encodes two glycoproteins, G1 and G2, and possibly a nonstructural protein, NS_M. A partial cDNA clone of the M segment which contains G1 and G2 glycoprotein coding sequences but lacks most of the NS_M sequences was inserted into the genome of vaccinia virus under the control of an early vaccinia promoter. Cells infected with the recombinant virus were found to synthesize two polypeptides with molecular weights of 65,000 (G1) and 55,000 (G2) that reacted specifically with antibody against PTV. Studies using indirect immunofluorescence microscopy revealed that these proteins accumulated intracellularly in the perinuclear region. The results of endoglycosidase H digestion of these glycoproteins suggested that both G1 and G2 glycoproteins were transported from the RER to the Golgi complex. These proteins were not chased out from the Golgi region during a 6-hr incubation in the presence of cycloheximide. Surface immune precipitation and ¹²⁵I-protein A binding assays also demonstrated that the majority of the G1 and G2 glycoproteins are retained intracellularly. These results indicate that the PTV glycoproteins contain the necessary information for retention in the Golgi apparatus. © 1988 Academic Press, Inc.

INTRODUCTION

The family Bunyaviridae comprises a large and heterogeneous group of arthropod-borne viruses with certain structural features in common (Bishop and Shope, 1979; Bishop *et al.*, 1980). The viruses are enveloped, and contain two surface glycoproteins, G1 and G2, an internal nucleocapsid-associated protein N, and a large internal protein (L) believed to be a transcriptase component. The nucleocapsid consists of three single-stranded RNA genome segments, L, M, and S. On the basis of genetic recombination and sequence analysis, it has been concluded that the S RNA encodes the nucleocapsid protein N and a nonstructural protein NS_S; the M RNA encodes two glycoproteins G1 and G2 and in some genera a nonstructural protein, NS_M; and the L RNA probably contains the information for the viral transcriptase (Bishop *et al.*, 1982; Bouloy *et al.*, 1984; Cabradilla *et al.*, 1983; Collett *et al.*, 1985; Eshita and Bishop, 1984; Fuller *et al.*, 1983; Ihara *et al.*, 1985; Lees *et al.*, 1986; Ronnholm and Pettersson, 1987; Schmaljohn *et al.*, 1987).

A characteristic feature of the members of the Bunyaviridae is their intracellular maturation by budding at smooth-surfaced membranes in the Golgi region (Murphy *et al.*, 1973). It has been reported for Uukuniemi virus, a member of the genus *Uukuvirus*, that both G1 and G2 glycoproteins accumulate in the Golgi region during virus infection (Kuismanen *et al.*, 1982, 1984).

It has also been demonstrated by using temperature-sensitive mutants that the intracellular accumulation of glycoproteins occurs in the absence of virus maturation (Gahmberg *et al.*, 1986). Intracytoplasmic virus budding has also been described for coronaviruses (Dubois-Dalcq *et al.*, 1982; Holmes *et al.*, 1981; Tooze *et al.*, 1984; Tooze and Tooze, 1985), flaviviruses (Leary and Blair, 1980), toroviruses (Fagerland *et al.*, 1986; Weiss and Horzinek, 1986), and rotaviruses (Altenburg *et al.*, 1980; Petrie *et al.*, 1984). It has been reported that the E1 glycoprotein of the coronaviruses, when expressed from cloned DNA, accumulated intracellularly (Machamer and Rose, 1987; Rottier and Rose, 1987). Furthermore, evidence has recently been reported which indicates that one of three hydrophobic membrane-spanning domains of this protein is responsible for retention in the Golgi complex (Machamer and Rose, 1987).

Punta Toro virus (PTV) is one of some 36 arthropod-borne viruses assigned to the *Phlebotomus* fever serogroup (*Phlebovirus* genus, Bunyaviridae, Bishop *et al.*, 1980) and has structural and morphological features similar to other members of the family Bunyaviridae. The S RNA of PTV has an unusual ambisense coding strategy. One protein (N) is coded in a subgenomic, viral-complementary S mRNA species and a second protein (NS_S) is coded by a viral sense mRNA (Ihara *et al.*, 1984). The M RNA segment possesses a single open reading frame in the viral-complementary sequence that is presumed to code for a polyprotein precursor.

¹ To whom requests for reprints should be addressed.

In addition to the G1 and G2 glycoprotein coding sequences, a sequence capable of coding a polypeptide of approximately 30 kDa was found preceding the G1 coding sequences, although the putative NS_M protein product corresponding to this sequence has not been identified either in virions or in infected cells. The estimated sizes of NS_M, G1, and G2 from the predicted amino acid sequence are 30,510, 60,897, and 55,005 Da, respectively (Ihara *et al.*, 1985). We have constructed vaccinia virus recombinants expressing the G1 and G2 glycoproteins of PTV, in order to study the possible existence of signals for intracellular retention in bunyavirus glycoproteins. We report here the expression of the two glycoproteins of PTV, and their localization in infected cells in the absence of other viral components.

MATERIALS AND METHODS

Cells and virus

CV-1, Vero, and HeLa T4⁺ cells (Maddon *et al.*, 1986) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn bovine serum. Human TK⁻143 cells maintained in the above medium supplemented with 25 μ g/ml of 5-bromo-2-deoxyuridine (5-BUDR). Vaccinia virus stocks were prepared and titrated by plaque assay in CV-1 cells. PTV stocks were prepared and titrated in Vero cells.

Construction of a vaccinia recombination plasmid containing the G1 and G2 glycoprotein genes

All DNA manipulations were carried out as described by Maniatis *et al.* (1982). Four cDNA clones, 5-79, 10-99, 6-37, and 4-148, which were previously used for sequence analysis, had been cloned into the *Pst*I site of pBR322 (Ihara *et al.*, 1985). By a series of restriction digestions and ligations with these clones, a full-length PTV M cDNA clone was constructed and inserted into pGEM-3 (Promega Biotech, Madison, WI), designated as pGEM-M. It was found, however, by analysis of protein synthesis in an *in vitro* transcription and translation system using pGEM-M and by subsequent sequence analysis that pGEM-M contained deletion mutations in the middle of the G1 glycoprotein coding region. It was also found that the sequences preceding the G1 coding sequences seem to have some inhibitory effects on growth of bacterial colonies. Because of these problems, most of the 5' sequence preceding the G1 coding region was removed by digestion with *Bal*/31 exonuclease. In order to repair the mutations, the *Nco*I-*Xba*I restriction fragment which contains the mutated region

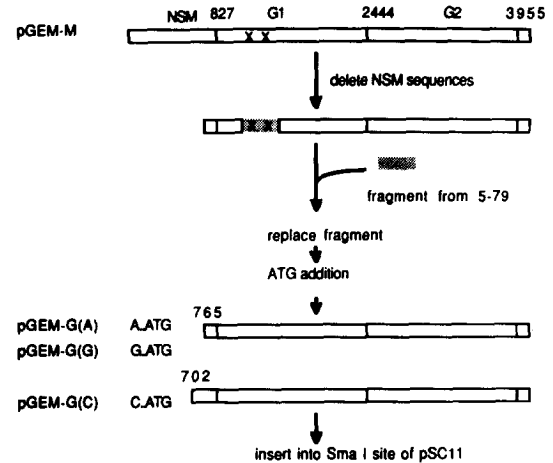


Fig. 1. Construction of PTV-M clones with shortened 5' end sequences. Most of the 5' sequence preceding the G1 coding sequence was removed from pGEM-M, which contains a full-length PTV M sequence with mutations, by *Bal*/31 digestion. The mutations were repaired by replacing the restriction fragment with the correct sequences. The translation initiation codon was generated for pGEM-G(A) and pGEM-G(G) by *Cla*I linker addition as described under Materials and Methods. The fragments containing PTV M segment sequences were excised from pGEM and inserted into the *Sma*I site of pSC11.

was replaced by the corresponding fragment from clone 5-79, one of the original clones used for sequence analysis (Fig. 1). After transformation and colony selection, two clones were obtained, one of which contained an M fragment beginning with nucleotide residue 702 that had an ATG at nucleotide residue 716, whereas the other contained a fragment beginning with residue 765 and lacked an initiation codon for synthesis of G1 and G2 glycoproteins. The clone containing an ATG codon in the region preceding the G1 coding sequences was designated as pGEM-G(C) since it possesses a C residue at the -3 position upstream from the translation initiation site. The clone which did not have an ATG sequence was further modified to introduce a translation initiation codon. After digestion with *Eco*RI and treatment with Klenow DNA polymerase, *Cla*I linkers were added at the ends of the DNA in order to provide an ATG sequence. This segment was then digested with *Cla*I and treated with Klenow polymerase following digestion with either *Pst*I or *Sal*I in order to remove plasmid sequences. These segments were inserted into either pGEM-3 digested with *Sal*I and treated with Klenow polymerase following *Pst*I digestion or pGEM-3 digested with *Xba*I and treated with Klenow polymerase following *Sal*I digestion. As a result, pGEM-G(A) which contains an A residue at the -3 position from the initiation codon, and pGEM-G(G) with a G residue at the -3 position, were obtained (Fig. 1).

The PTV glycoprotein gene was excised by *Bam*HI from the three clones, pGEM-G(C), pGEM-G(A), and pGEM-G(G), treated with Klenow polymerase, and inserted into the *Sma*I site of the pSC11 vaccinia recombination plasmid (Chakrabarti *et al.*, 1985).

Construction of a vaccinia recombination plasmid containing the influenza HA gene

The cDNA clone of the WSN-HA gene in pBR322 was kindly provided by Dr. Debi Nayak. The HA gene was excised from pBR322 and recloned into the *Pst*I site of pUC13 (Stephens *et al.*, 1986). The plasmid was linearized by *Bam*HI digestion, treated with *Bal*31 exonuclease to remove dG and dC homopolymeric tails at the 5' end, treated with Klenow DNA polymerase, and then digested with *Hind*III. The HA gene fragment was separated from the pUC13 vector, inserted into *Hind*III-*Hinc*II-digested pGEM-3, excised by *Xba*I-*Hind*III digestion, treated with Klenow DNA polymerase, and inserted into the *Sma*I site of pSC11.

Isolation of vaccinia virus recombinants

For isolation of vaccinia recombinants, CV-1 cells were infected with vaccinia virus (strain IHD-J) at a multiplicity of infection (m.o.i.) of 0.05. At 2 hr after infection, cells were transfected with a calcium phosphate precipitate of 10 μ g of plasmid and 15 μ g of salmon sperm DNA/ml of HEPES-buffered saline (Graham and Van der Eb, 1973). Stocks of TK⁻ vaccinia virus were prepared in TK⁻143 cells (Smith and Moss, 1983). To select for recombinants, TK⁻143 cells were infected with 50–100 PFU of TK⁻ vaccinia virus in the presence of BUdR (25 μ g/ml). At 48 hr after infection, the monolayers were overlaid with 1% low-melting-point agarose containing 300 μ g/ml of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). At 4–6 hr, blue plaques were picked and further purified by two additional rounds of plaque purification.

Antisera and immunoprecipitation of radiolabeled proteins

Hyperimmune mouse ascitic fluids specific for PTV and monoclonal antibodies against G1 and G2 glycoproteins were kindly provided by Drs. J. F. Smith and D. Pifat (USAMRIID, Fort Detrick, MD). Rabbit antiserum against A/WSN/33 influenza virus was prepared as described elsewhere (Roth and Compans, 1980). CV-1 cells or HeLa T4⁺ cells were infected with vaccinia virus recombinants or PTV at an m.o.i. of 5 to 10. At 16 hr p.i., cells were washed with phosphate-buffered saline (PBS) and incubated in methionine-free medium for 2

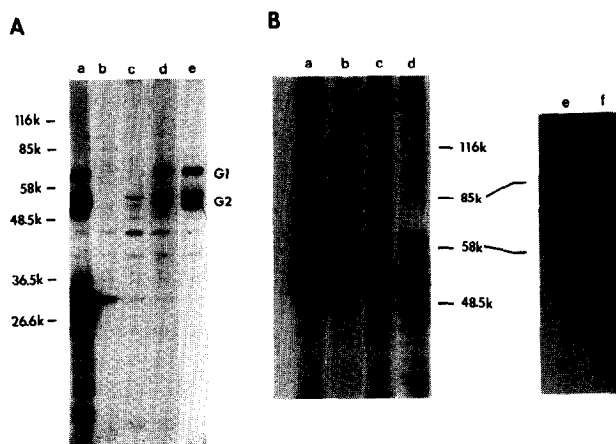


Fig. 2. (A) SDS-PAGE analysis of PTV-specific polypeptides expressed in vaccinia recombinant-infected cells. CV-1 cells were infected with PTV (lane a), wild-type vaccinia virus (lane b), VV-G(C) (lane c), VV-G(G) (lane d), or VV-G(A) (lane e). Samples were immunoprecipitated as described under Materials and Methods and analyzed on 10% polyacrylamide gels. (B) TM and/or PMSF treatment of VV-G-infected HeLa T4⁺ cells. Cells infected with PTV (lanes a and c), VV-G (lanes b and d), or VV-HA (lanes e and f) were incubated in medium containing 1.0 mM/ml of PMSF in the presence (lanes b, d, and f) or absence (lanes a, c, and e) of 1.0 μ g/ml of TM prior to and during the [³⁵S]methionine labeling. Cell lysates were immunoprecipitated and analyzed by SDS-PAGE.

hr. At 18 hr p.i., cells were labeled with [³⁵S]methionine (100 μ Ci/ml) in methionine-free medium for 1 hr. For pulse-chase experiments, cells were pulsed for 10 min and then chased in Eagle's medium containing 10 mM methionine for an appropriate period. Cells were then washed three times in ice-cold PBS and lysed with 0.3 ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 20 mM EDTA). Nuclei were removed by centrifugation at 13,000 *g* for 5 min at 4°. The cell lysates were incubated with 5 μ l of mouse ascites against PTV for 4 hr at 4° with constant mixing. For immunoprecipitation of cell-surface antigens, antibody was added before cells were lysed and incubated for 30 min on ice. After washing to remove unbound antibody, radiolabeled polypeptides were immunoprecipitated with protein A-Sepharose CL-4B (Pharmacia, Inc., Piscataway, NJ). The precipitates were washed three times with cold lysis buffer, resuspended in sample buffer, boiled for 5 min, and analyzed on 10% SDS-polyacrylamide gels (Laemmli, 1970).

Effect of tunicamycin

HeLa T4⁺ cells were infected with PTV or vaccinia recombinants at an m.o.i. of 10. After 16 hr p.i., cells were incubated in methionine-free medium containing 1.0 mM of phenylmethylsulfonyl fluoride (PMSF)

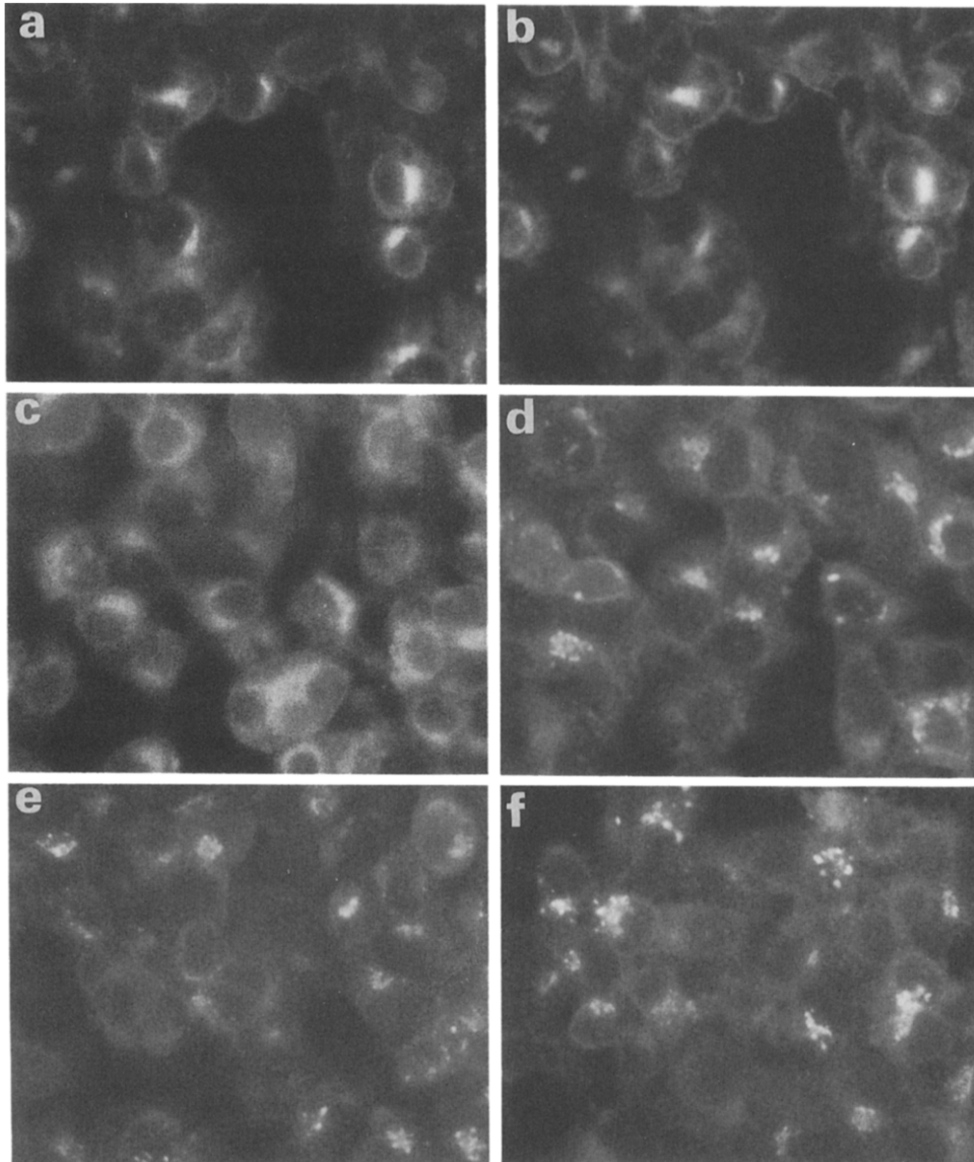


FIG. 3. Localization of PTV glycoproteins in VV-G-infected HeLa T4⁺ cells by indirect immunofluorescence microscopy. At 18 hr p.i., VV-G-infected cells were fixed with ethanol containing 5% acetic acid and indirectly stained with anti-PTV hyperimmune mouse ascites fluid by using fluorescein-conjugated goat anti-mouse IgG (a). The same cells in (a) were doubly stained by using rhodamine-conjugated wheat germ agglutinin (b). The cells were treated with cycloheximide (50 µg/ml) at 16 hr p.i. for 6 hr. VV-G-infected cells before treatment (c) and after 6 hr exposure with drug (d, e, and f) were stained with anti-PTV mouse ascites fluid (c and d), anti-G1 monoclonal antibody (e), or anti-G2 monoclonal antibody (f).

(Sigma, St. Louis, MO) in the presence or absence of 1.0 µg/ml of tunicamycin (TM) (Calbiochem, La Jolla, CA) for 2 hr. Samples were then labeled with [³⁵S]methionine for 1 hr and examined by immunoprecipitation as described. During the labeling period, PMSF and TM were maintained in medium of treated cells.

Indirect immunofluorescence

HeLa T4⁺ cells on glass coverslips were infected with vaccinia virus, recombinant vaccinia viruses, or

PTV at an m.o.i. of 10. At appropriate times after infection, cells were washed with PBS and fixed with ethanol containing 5% acetic acid for 20 min at -20° or with 2% formaldehyde for 10 min at room temperature. Cells were then washed with PBS and reacted with antibodies specific for PTV for 30 min followed by a fluorescein-conjugated goat anti-mouse IgG or a fluorescein-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL). Infected cells were also labeled simultaneously with rhodamine-conjugated wheat germ agglutinin (E-Y

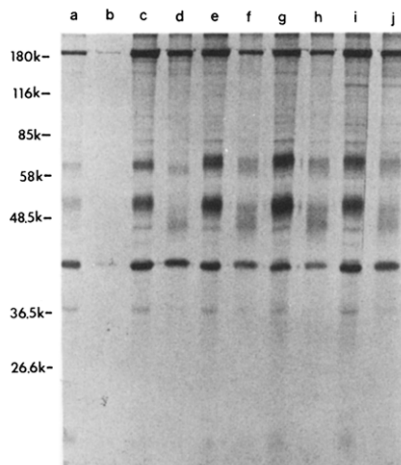


Fig. 4. Endo H digestion of the G1 and G2 glycoproteins produced by VV-G. At 18 hr p.i., VV-G-infected cells were pulse labeled for 10 min (lanes a and b) and chased for 60 min (lanes c and d), 120 min (lanes e and f), 180 min (lanes g and h) or 240 min (lanes i and j). Cells were then lysed and immunoprecipitated. Half of each sample was further treated by endo H at 37° for 16 hr (lanes b, d, f, h, and j). All samples were then analyzed on 10% SDS-PAGE.

Laboratories, Inc., San Mateo, CA) in some experiments. The cells were washed with PBS, mounted, and observed with a Nikon Optiphot microscope equipped with a modified B2 cube.

Endoglycosidase H digestion

Immunoprecipitated samples were boiled for 5 min in SDS-PAGE sample buffer and the protein A-Sepharose was removed. The supernatants were diluted 15-fold with 0.1 M sodium acetate, pH 6.0, and incubated for 16 hr at 37° in the presence of 8 mU/ml of endoglycosidase H (endo H) (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and 1 mM of PMSF. Samples were then precipitated with 10% TCA for 1 hr at 0°, washed with cold ethanol/ether (1:1), and resuspended for SDS-PAGE analysis.

¹²⁵I-protein A binding assay

To quantitate antigen expressed on cell surface, surface radioimmunoassays were performed by a method described by Stephens *et al.* (1986). HeLa T4⁺ cells on glass coverslips were infected with vaccinia virus or the vaccinia virus recombinant at an m.o.i. of 10. At appropriate times after infection, cells were washed three times with PBS/1% bovine serum albumin (BSA), overlaid with 10 μ l of antibody diluted 1:10 in PBS/1% BSA, and incubated for 30 min at 4°. Cells were then washed three times with PBS/1% BSA and incubated with 200,000 cpm of ¹²⁵I-protein A (Amersham, Arlington

Heights, IL) prepared in PBS/1% BSA for 30 min at room temperature. The label was removed, the cells were washed three times with PBS/1% BSA, and the bound radioactivity was determined. Nonspecific binding of the ¹²⁵I-protein A to cells infected with wild-type vaccinia virus was determined for each antisera and subtracted from each value.

RESULTS

Identification of glycoproteins produced from vaccinia virus recombinants in CV-1 cells

To compare the levels of PTV glycoprotein synthesis, monolayers of CV-1 cells were infected with wild-type vaccinia virus, PTV, or the vaccinia recombinants described above which contain the PTV glycoprotein gene with the different sequences in the translation initiation region. At 18 hr p.i., cells were labeled for 1 hr with [³⁵S]methionine, harvested, and immunoprecipitated. Two polypeptides, which have electrophoretic mobility similar to those of the G1 and G2 glycoproteins synthesized in PTV-infected cells, were precipitated from cells infected with vaccinia recombinants using antibody specific for PTV (Fig. 2A). The polypeptide corresponding to the G2 glycoprotein occasionally appeared as a doublet. The appearance of the lower band was not consistently observed, and it may be a differently glycosylated form of the G2 glycoprotein. A similar observation of the synthesis of a heterogeneous Hantaan (HTN) G1 glycoprotein was reported by Pensiero *et al.* (1988). The level of protein synthesis was much higher for the recombinants which contain G or A at the -3 translation initiation position than the one which contained C. Therefore, one of the recombinants which contains G at the -3 position was chosen as a prototype (designated VV-G) and used for further experiments.

The effect of tunicamycin (TM) on the synthesis of the proteins was also examined as shown in Fig. 2B. At 16 hr p.i., cells infected with PTV or recombinants were exposed to medium containing TM for 2 hr. Cells were then labeled with [³⁵S]methionine for 1 hr, lysed, and immunoprecipitated. The G1 glycoproteins synthesized from the VV-G recombinant, in either the presence or absence of TM, showed slightly slower mobility on the gel than those synthesized in PTV-infected cells. The G2 glycoprotein appeared as multiple bands in the presence of TM, which may indicate a partial inhibition of glycosylation. The amount of G1 and G2 glycoproteins detected in the presence of TM was significantly lower than that in the absence of TM. In contrast, under the same condition, the glycosylation of

influenza HA glycoprotein was completely inhibited and similar amounts of both glycosylated and unglycosylated forms of HA were detected. When a higher concentration of TM (1.5 $\mu\text{g/ml}$) was used, neither the G1 nor G2 glycoprotein was detected (data not shown). Therefore, the unglycosylated forms of G1 and G2 glycoproteins appear to be relatively unstable and quickly degraded even in the presence of PMSF.

Localization of the glycoproteins in HeLa T4⁺ cells infected with recombinants

The intracellular localization of the expressed glycoproteins was examined in HeLa T4⁺ cells because this cell line shows minimal cytopathic effect with vaccinia virus infection when compared to most other cell lines, and is therefore well suited for studies of protein localization (R. Owens and R. Compans, submitted for publication). HeLa T4⁺ cells were infected with the VV-G recombinant, and at 18 hr p.i., cells were fixed and examined using indirect immunofluorescence microscopy. The proteins expressed from VV-G were localized in a perinuclear region which coincided with the region stained by wheat germ agglutinin, a marker for the Golgi complex (Figs. 3a and b). Recombinant VV-G-infected cells were also treated with cycloheximide to determine whether G1 and G2 glycoproteins are chased out from the Golgi region. As shown in Fig. 3, both glycoproteins were retained in the Golgi region after a 6-hr chase with cycloheximide. The accumulation of these proteins in a localized region of the cytoplasm was much more apparent in cycloheximide-treated cells (d, e, and f) compared to the cells before treatment (c).

In order to further investigate the intracellular location of the glycoproteins, the processing of their oligosaccharide side chains was studied by treatment with endo H. After pulse labeling of VV-G-infected cells and various chase periods, proteins were immunoprecipitated and digested with endo H (Fig. 4). The G1 synthesized from the VV-G recombinant acquired endo H resistance within a 2-hr chase period, suggesting that it contains complex-type oligosaccharide side chains. The G2 glycoprotein, on the other hand, appears to contain both high mannose-type and complex- or intermediate-type of sugar moieties. These results indicate that the G1 and G2 glycoproteins are transported from the RER to the Golgi complex where the maturation of oligosaccharides takes place.

No significant amount of surface expression of viral glycoproteins was observed on cells infected with PTV or VV-G when examined by surface immunofluorescence (Figs. 5e and f). In contrast, the vaccinia recom-

binant expressing the influenza HA glycoprotein is shown as a control which exhibits typical surface protein expression (Fig. 5h).

The lack of significant surface expression of the PTV glycoproteins was also examined by two biochemical approaches. At 18 hr p.i., cells infected with the VV-G recombinant were pulsed for 10 min with [³⁵S]methionine, chased with medium containing excess cold methionine for 30, 60, 120, and 180 min, and the presence of proteins on cell surfaces was analyzed by surface immune precipitation. As shown in Fig. 6, lanes f–j, only faint bands were seen in the surface immunoprecipitate. No increase in the intensity of G1 or G2 proteins was seen during the chase, indicating that the proteins are not being transported to the cell surface during this time period. It was also confirmed that the ascitic fluids specific for PTV possess virus neutralizing activity, and hence have the ability to react with extracellular forms of PTV glycoproteins (data not shown). In order to determine whether the PTV glycoproteins are secreted, supernatants of VV-G-infected cell cultures were examined. At 16 hr p.i., infected cells were labeled with [³⁵S]methionine for 6 hr, and proteins present in culture media were immunoprecipitated and analyzed by SDS-PAGE. No detectable amount of the G1 or G2 glycoproteins was found in the medium (data not shown).

Cell-surface expression was also quantitated by ¹²⁵I-protein A binding assays at different time points of infection. The vaccinia recombinant expressing HA was again used as a control for surface expression. As shown in Fig. 7, HA was detectable on the cell surface at 8 hr p.i. and the amount increased until 16 hr p.i. In contrast, PTV glycoproteins were barely detectable until 16 hr p.i., and the level of maximum ¹²⁵I-protein A binding was significantly lower than that observed with HA. Throughout the experiment, no significant cytopathic effect was observed. The percentage of live cells in recombinant-infected cultures determined by trypan blue staining was the same as in uninfected cells.

DISCUSSION

We have constructed vaccinia virus recombinants containing a partial cDNA clone of the M genome segment of PTV, which encodes the G1 and G2 glycoproteins. Although a complete M segment clone was initially obtained, because of a mutation in the G1 glycoprotein gene and apparent inhibitory effects of the sequence preceding the G1 glycoprotein gene on bacterial growth, which interfered with attempts to repair mutations, most of the 5' sequences preceding the G1

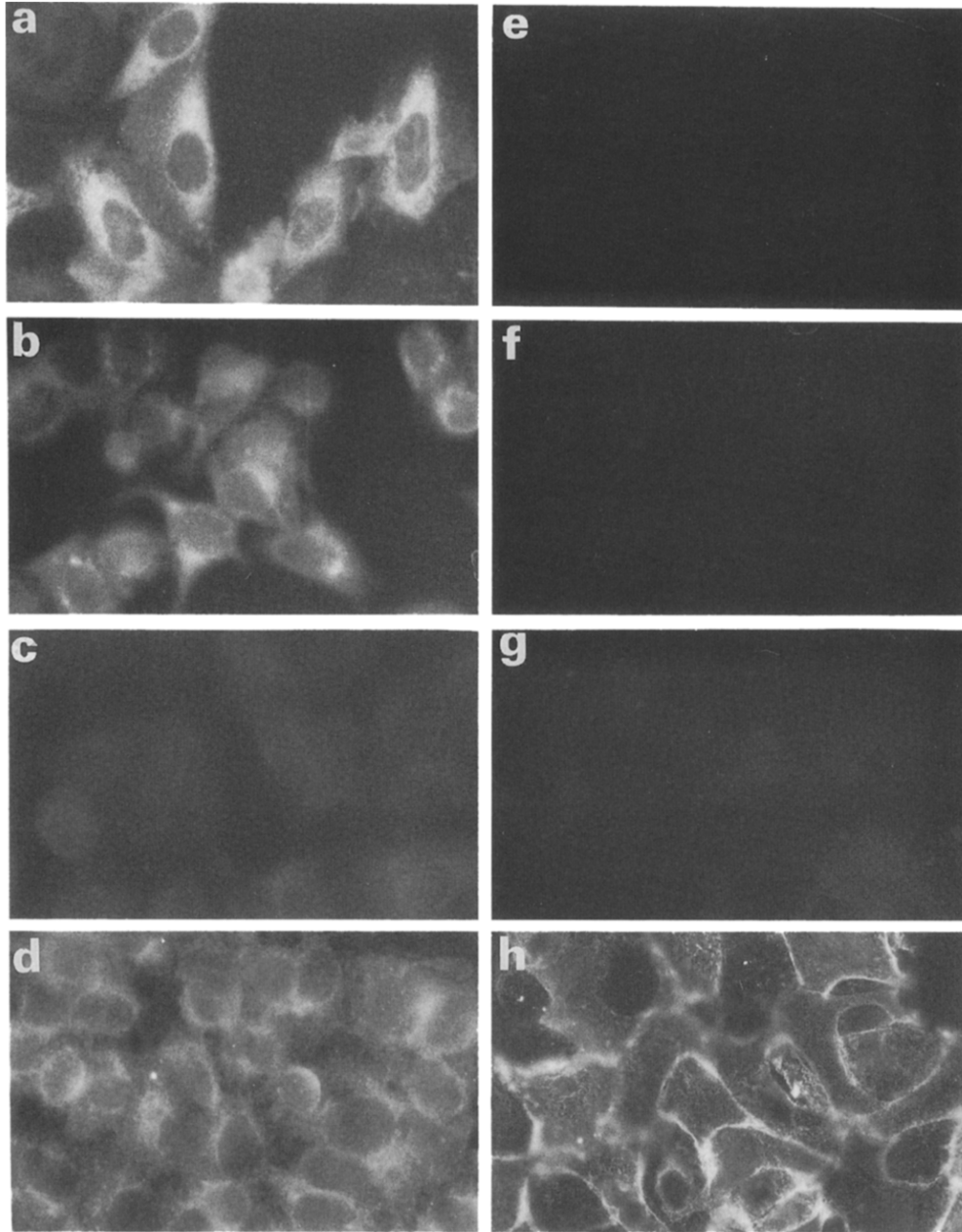


FIG. 5. Lack of surface expression of PTV glycoproteins. Cells were infected with PTV (a and e), VV-G (b and f), vaccinia virus (c and g), or VV-HA (d and h). At 18 hr p.i. cells were fixed either with ethanol containing 5% acetic acid (a, b, c, and d) or with 2% formaldehyde (e, f, g, and h). Left panels show intracellular immunofluorescence and right panels show surface immunofluorescence.

coding sequences had to be eliminated to obtain glycoprotein expression. This recombinant virus, VV-G, efficiently produced two polypeptides that were recognized by antibody against PTV.

It has been suggested that the two glycoproteins of bunyaviruses, including PTV, are derived from a large precursor protein following proteolytic cleavage (Collett *et al.*, 1985; Eshita and Bishop; 1984, Ihara *et al.*, 1985). Although the precursor molecule for the glycoproteins has never been identified in virus-infected

cells, it was produced in an *in vitro* translation system using mRNA isolated from Uukuniemi virus-infected cells (Ulmanen *et al.*, 1981). The VV-G recombinant produced two glycoproteins similar in size to G1 and G2 from PTV-infected cells, suggesting that the cleavage between G1 and G2 occurs correctly. It was observed, however, by using tunicamycin (TM) that the unglycosylated form of G1-synthesized from recombinants had a slightly higher molecular weight than that from PTV-infected cells. Since only one predicted gly-

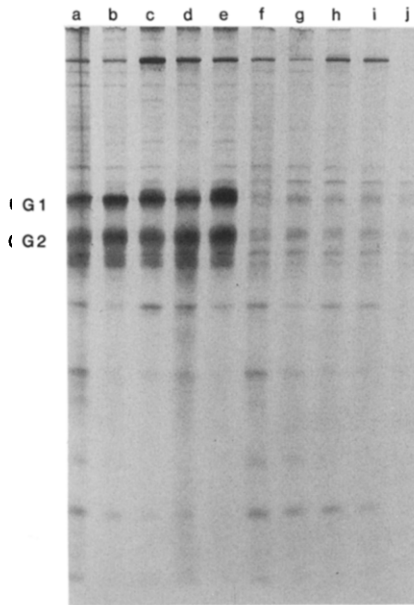


FIG. 6. Assay of PTV glycoproteins on the cell surface by immune precipitation. At 18 hr p.i., HeLa T4⁺ cells infected with VV-G were labeled with [³⁵S]methionine for 10 min (lanes a and f) and chased for 30 min (lanes b and g), 60 min (lanes c and h), 120 min (lanes d and i), or 180 min (lanes e and j) in medium containing 10 mM unlabeled methionine. Cells were lysed and reacted with anti-PTV ascites fluid (lanes, a, b, c, d, and e) or treated with anti-PTV ascites fluid and then lysed (lanes f, g, h, i, and j). Following immunoprecipitation, radiolabeled polypeptides were analyzed by SDS-PAGE.

cosylation site was found in the G1 glycoprotein sequence including the extra short sequences at the 5' end in VV-G (Ihara *et al.*, 1985), it is likely that the decrease in the size of G1 glycoproteins in the presence of TM reflects the complete inhibition of glycosylation. Also, the apparent molecular weight of the unglycosylated form of G1 glycoprotein in PTV-infected cells corresponded to the size predicted from sequence analysis (60,000 Da). The observed difference in size (2–3 kDa) between unglycosylated G1 of PTV and of VV-G may, therefore, correspond to the extra 24 amino acids preceding the G1 glycoprotein, which are derived from the putative NS_M coding region. In this case, cleavage between G1 and the partial NS_M sequence may not take place. Although there seems to be a consensus sequence after alanine for the potential cleavage sites in the putative precursor protein for PTV as well as Rift Valley Fever virus (RVFV) (Ihara *et al.*, 1985; Collett *et al.*, 1985), the present results indicate that an additional factor such as secondary structure of the protein may also be necessary for proper cleavage. Uncleaved precursor proteins were not detected in either PTV- or recombinant-infected cells in the presence of protease inhibitors and/or TM. Therefore, glycosylation is apparently not essential for cleavage.

The PTV glycoproteins seem to contain the necessary information for intracellular localization as well. The majority of proteins produced from the VV-G, recombinant remain intracellularly throughout the infection period. The intracellular location of these glycoproteins was further examined by digestion of proteins with endo H. It is generally considered that the time required for the acquisition of endo H resistance corresponds to the transport time from the RER to the Golgi complex (Strous and Lodish, 1980). The finding of endo H resistant forms of G1 and G2 glycoproteins indicates that these glycoproteins are transported from the RER to the Golgi complex. Furthermore, after 6 hr treatment with cycloheximide, both glycoproteins were still localized in the Golgi region by immunofluorescence, indicating that these proteins are retained in the Golgi complex. At late times postinfection, small amounts of proteins were detected on the cell surface by ¹²⁵I-protein A binding assays but not by immunofluorescence. Since these glycoproteins were retained in the Golgi region for 6 hr in the presence of cycloheximide, any transport from the Golgi complex to the cell surface must be extremely slow if it takes place. Cell-surface expression of small amounts of glycoproteins has also been reported for other bunyaviruses (Kuismanen *et al.*, 1982; Madoff and Lenard, 1982; Gahmberg *et al.*, 1986). However, these proteins may be present on cell-associated virions (Smith and Pifat, 1982).

Recently, it has been reported that glycoproteins of Hantaan virus (HTN), *Hantavirus* genus of bunyaviridae, when expressed from a cloned M segment gene, also accumulate intracellularly (Pensiero *et al.*, 1988;

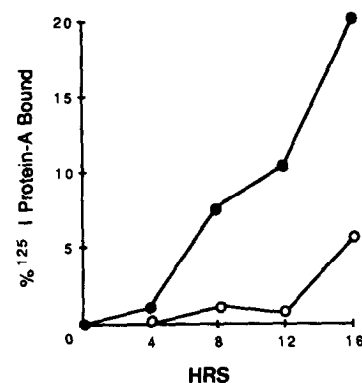


FIG. 7. ¹²⁵I-protein A binding assays of HeLa T4⁺ cells infected with VV-G or VV-HA. HeLa T4⁺ cells were infected with VV-G (○), VV-HA (●), or wild-type vaccinia virus. At 4-hr intervals, cells were used in ¹²⁵I-protein A binding assays. Background levels of ¹²⁵I-protein A binding determined by infection with vaccinia virus were subtracted from all plotted values. Infection with vaccinia virus yielded background levels of protein A binding that were 2.0–3.5% for anti-PTV and 4–6% for anti-influenza antiserum.

Schmaljohn *et al.*, 1987). Unlike PTV or RVFV, the HTN M genome segment lacks the 5' sequences which may encode another protein. Instead it possesses 18 amino acids following the first ATG of the M segment, and preceding the G1 glycoprotein, which may simply act as a signal peptide. These results, as well as the present observations, indicate that the glycoproteins of bunyavirus are synthesized, processed, and localized properly without a requirement for any other viral components, such as viral nucleocapsid or virus-coded nonstructural proteins.

The precise location in the glycoproteins of the signals for intracellular retention remains to be determined. A recent study of the E1 glycoprotein of a coronavirus (Machamer and Rose, 1987) indicated a role of hydrophobic sequences in protein retention. Deletion of one of the hydrophobic membrane spanning domains of these glycoproteins, which are normally retained at intracellular membranes, resulted in their secretion or transport to the surface. It will be of interest to carry out similar studies to determine the precise retention signal for PTV glycoproteins.

ACKNOWLEDGMENTS

This study was supported by Grant AI 12680 from the National Institute of Allergy and Infectious Diseases. We thank Drs. Jonathan Smith and Dominique Pifat for providing antibodies, and Dr. Marc Collett for helpful suggestions.

REFERENCES

- ALTENBURG, B. C., GRAHAM, D. Y., and ESTES, M. K. (1980). Ultrastructural study of rotavirus replication in cultured cells. *J. Gen. Virol.* **46**, 75–85.
- BISHOP, D. H. L., CALISHER, C. H., CASALS, J., CHUMAKOV, M. P., GAIDAMOVICH, S. Y., HANNOUN, C., LVOV, D. K., MARSHALL, I. D., OKER-BLOM, N., PETTERSSON, R. F., PORTERFIELD, J. S., RUSSELL, P. K., SHOPE, R. E., and WESTAWAY, E. G. (1980). Bunyaviridae. *Intervirology* **14**, 125–143.
- BISHOP, D. H. L., GOULD, K. G., AKASHI, H., and CLERX-VAN HAASTER, C. M. (1982). The complete sequence and coding content of snowshoe hare bunyavirus small (S) viral RNA species. *Nucleic Acids Res.* **10**, 3703–3713.
- BISHOP, D. H. L., and SHOPE, R. E. (1979). Bunyaviridae. In "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, Eds.), Vol. 14, pp. 1–156. Plenum, New York.
- BOULOY, M., VIALAT, P., GIRARD, M., and PARDIGON, N. (1984). A transcript from the S segment of the Germiston bunyavirus is uncapped and codes for the nucleoprotein and a nonstructural protein. *J. Virol.* **49**, 717–723.
- CABRADILLA, C. D., HOLLOWAY, B. P., and OBIJESKI, J. F. (1983). Molecular cloning and sequencing of the La Crosse virus S RNA. *Virology* **128**, 463–468.
- CHAKRABARTI, S., BRECHLING, K., and MOSS, B. (1985). Vaccinia virus expression vector: Coexpression of β -galactosidase provides visual screening of recombinant virus plaques. *Mol. Cell. Biol.* **5**, 3403–3409.
- COLLETT, M. S., PURICCHIO, A. F., KEEGAN, K., FRAZIER, S., HAYS, W., ANDERSON, D. K., PARKER, M. D., SCHMALJOHN, C., SCHMIDT, J., and DALRYMPLE, J. M. (1985). Complete nucleotide sequence of the M RNA segment of Rift Valley fever virus. *Virology* **144**, 228–245.
- DUBOIS-DALCO, M. E., DOLLER, E. W., HASPEL, M. V., and HOLMES, K. V. (1982). Cell tropism and expression of mouse hepatitis virus (MHV) in mouse spinal cord cultures. *Virology* **119**, 317–331.
- ESHITA, Y., and BISHOP, D. H. L. (1984). The complete sequence of the M RNA of snowshoe hare bunyavirus reveals the presence of internal hydrophobic domains in the viral glycoprotein. *Virology* **137**, 227–240.
- FAGERLAND, J. A., POHLEUZ, J. F. L., and WOODE, G. N. (1986). A morphological study of the replication of Breda virus (proposed family Toroviridae) in bovine intestinal cells. *J. Gen. Virol.* **67**, 1294–1304.
- FULLER, F., BHOWN, A. S., and BISHOP, D. H. L. (1983). Bunyavirus nucleoprotein, N, and a non-structural protein, NS, are coded by overlapping reading frames in the S RNA. *J. Gen. Virol.* **64**, 1705–1714.
- GAHMBERG, N., KUISMANEN, E., KERANEN, S., and PETTERSSON, R. F. (1986). Uukuniemi virus glycoproteins accumulate in and cause morphological changes of the Golgi complex in the absence of virus maturation. *J. Virol.* **57**, 899–906.
- GRAHAM, F. L., and VAN DER EB, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456–467.
- HOLMES, K. V., DOLLER, E. W., and STURMAN, L. S. (1981). Tunica-mycin resistant glycoprotein of a coronavirus glycoprotein: demonstration of a novel type of viral glycoprotein. *Virology* **115**, 334–344.
- IHARA, T., AKASHI, H., and BISHOP, D. H. L. (1984). Novel coding strategy (ambisense genomic RNA) revealed by sequence analyses of Punta Toro phlebovirus S RNA. *Virology* **136**, 293–306.
- IHARA, T., SMITH, J., DALRYMPLE, J. M., and BISHOP, D. H. L. (1985). Complete sequence of the glycoproteins and M RNA of Punta Toro phlebovirus compared to those of Rift Valley fever virus. *Virology* **144**, 246–259.
- KAKACH, L. T., WASMOEN, T. L., and COLLETT, M. S. (1988). Rift Valley fever virus M segment: Use of recombinant vaccinia virus to study *Phlebovirus* gene expression. *J. Virol.* **62**, 826–833.
- KUISMANEN, E., BANG, B., HURME, M., and PETTERSSON, R. F. (1984). Uukuniemi virus maturation: immunofluorescence microscopy with monoclonal glycoprotein-specific antibodies. *J. Virol.* **51**, 137–146.
- KUISMANEN, E., HEDMAN, K., SARASTE, J., and PETTERSSON, R. F. (1982). Uukuniemi virus maturation: Accumulation of virus particles and viral antigens in the Golgi complex. *Mol. Cell. Biol.* **2**, 1444–1458.
- LAEMMLI, U. K. (1970). Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685.
- LEARY, K., and BLAIR, C. D. (1980). Sequential events in the morphogenesis of Japanese encephalitis virus. *J. Ultrastruct. Res.* **72**, 123–129.
- LEES, J. F., PRINGLE, C. R., and ELLIOTT, R. M. (1986). Nucleotide sequence of the bunyamwera virus M RNA segment: Conservation of structural features in the bunyavirus glycoprotein gene product. *Virology* **148**, 1–14.
- MACHAMER, C. E., and ROSE, J. K. (1987). A specific transmembrane domain of a coronavirus E1 glycoprotein is required for its retention in the Golgi region. *J. Cell Biol.* **105**, 1205–1214.
- MADDON, P. J., DALGLEISH, A. G., MCDUGAL, J. S., CLAPHAM, P. R., WEISS, R. A., and AXEL, R. (1986). The T4 gene encodes the AIDS

- virus receptor and is expressed in the immune system and the brain. *Cell* **47**, 333–348.
- MADOFF, D. H., and LENARD, J. (1982). A membrane glycoprotein that accumulates intracellularly: Cellular processing of the large glycoprotein of LaCrosse virus. *Cell* **28**, 821–829.
- MANIATIS, T., FRITSCH, E. F., and SUMBROOK, J. (1982). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- MURPHY, F. A., HARRISON, A. K., and WHITFIELD, S. G. (1973). Bunyaviridae: Morphologic and morphogenetic similarities of bunyamwera serologic supergroup viruses and several other arthropod-borne viruses. *Intervirology* **1**, 297–316.
- PENSIERO, M. N., JENNINGS, G. B., SCHMALJOHN, C. S., and HAY, J. (1988). Expression of Hantaan virus M genome segment by using vaccinia virus recombinant. *J. Virol.* **62**, 696–702.
- PETRIE, B. L., GREENBERG, H. B., GRAHAM, D. Y., and ESTES, M. K. (1984). Ultrastructural localization of rotavirus antigens using colloidal gold. *Virus Res.* **1**, 133–152.
- RONNHOLM, R., and PETERSSON, R. F. (1987). Complete nucleotide sequence of the mRNA segment of Uukuniemi virus encoding the membrane glycoproteins G1 and G2. *Virology* **160**, 191–202.
- ROTH, M. G., and COMPANS, R. W. (1980). Antibody-resistant spread of vesicular stomatitis virus infection in cell lines of epithelial origin. *J. Virol.* **35**, 547–550.
- ROTTIER, P. J. M., and ROSE, J. K. (1987). Coronavirus E1 glycoprotein expressed from cloned cDNA localize in the Golgi region. *J. Virol.* **61**, 2042–2045.
- SCHMALJOHN, C. S., SCHMALJOHN, A. L., and DALRYMPLE, J. M. (1987). Hantaan virus M RNA: Coding strategy, nucleotide sequence, and gene order. *Virology* **157**, 31–39.
- SMITH, G. L., and MOSS, B. (1983). Infectious poxvirus have capacity for at least 25,000 base pairs of foreign DNA. *Gene* **25**, 21–28.
- SMITH, J. F., and PIFAT, D. Y. (1982). Morphogenesis of sandfly fever viruses (Bunyaviridae family). *Virology* **121**, 61–81.
- STEPHENS, E. B., COMPANS, R. W., EARL, P., and MOSS, B. (1986). Surface expression of viral glycoproteins is polarized in epithelial cells infected with recombinant vaccinia viral vectors. *EMBO J.* **5**, 237–245.
- STROUS, G. J. A. M., and LODISH, H. F. (1980). Intracellular transport of secretory and membrane proteins in hepatoma cells infected with vesicular stomatitis virus. *Cell* **22**, 709–717.
- TOOZE, J., and TOOZE, S. A. (1985). Infection of AtT20 murine pituitary tumor cells by mouse hepatitis virus strain A59: Virus budding is restricted to the Golgi region. *Eur. J. Cell Biol.* **37**, 203–212.
- TOOZE, J., TOOZE, S. A., and WARREN, G. (1984). Replication of coronavirus MHV-A59 in Sac (–) cells: Determination of the first site of budding of progeny virions. *Eur. J. Cell Biol.* **33**, 281–293.
- ULMANEN, I. L., SEPPALA, P., and PETERSSON, R. F. (1981). *In vitro* translation of Uukuniemi virus-specific RNAs: Identification of a nonstructural protein and a precursor to the membrane glycoproteins. *J. Virol.* **37**, 72–79.
- WEISS, M., and HORZINEK, M. C. (1986). Morphogenesis of Berne virus (proposed family Toroviridae). *J. Gen. Virol.* **67**, 1305–1314.