JOURNAL OF VIROLOGY, July 1992, p. 4233–4241
0022-538X/92/074233-09$02.00/0

Vol. 66, No. 7

Association of the Nonstructural Protein NS_s of Uukuniemi Virus with the 40S Ribosomal Subunit

JAN FREDRIK SIMONS,* ROBERT PERSSON, AND RALF F. PETTERSSON

Stockholm Branch, Ludwig Institute for Cancer Research, Box 60202, S-10401 Stockholm, Sweden

Received 21 January 1992/Accepted 14 April 1992

The small RNA segment (S segment) of Uukuniemi (UUK) virus encodes two proteins, the nucleocapsid protein (N) and a nonstructural protein (NS_s), by an ambisense strategy. The function of NS_s has not been elucidated for any of the bunyaviruses expressing this protein. We have now expressed the N and NS_s proteins in Sf9 insect cells by using the baculovirus expression system. High yields of both proteins were obtained. A monospecific antibody was raised against gel-purified NS_s and used to study the synthesis and localization of the protein in UUK virus-infected BHK21 cells. While the N protein was detected as early as 4 h postinfection (p.i.), NS_s was identified only after 8 h p.i. Both proteins were still synthesized at high levels at 24 h p.i. The half-life of NS_s was about 1.5 h, while that of the N protein was several hours. Sucrose gradient fractionation of [^{35}S]methionine-labeled detergent-solubilized extracts of infected BHK21 cells indicated that NS_s was firmly associated with the 40S ribosomal subunit. This association took place shortly after translation and was partially resistant to 1 M NaCl. NS_s expressed by using the T7 vaccinia virus expression system, as well as in vitro-translated NS_s, was also associated with the 40S subunit. In contrast, in vitro-translated N protein was found on top of the gradient. Immunolocalization of NS_s, in UUK virus-infected cells, by using an affinity-purified antibody showed a granular cytoplasmic staining. A very similar pattern was seen for cells expressing NS_s from a cDNA copy by using a vaccinia virus expression system. No staining was observed in the nuclei in either case. Furthermore, NS_s was found neither in virions nor in nucleocapsids isolated from infected cells. In vivo labeling with $^{32}P_i$ indicated that NS_s is not phosphorylated. The possible function of NS_s is discussed in light of these results.

The *Bunyaviridae* family consists of a large group of arthropod-borne viruses, which are classified into five separate genera. The viruses in four of the genera, i.e., bunyaviruses, phleboviruses, nairoviruses, and hantaviruses, propagate in animal hosts, whereas the recently included tomato spotted wilt virus, the only member of the *Tospovirus* genus, is a plant virus (3).

The bunyavirus genome consists of three single-stranded RNA segments of negative or ambisense polarity. The RNAs are associated with the nucleocapsid protein (N) and the RNA polymerase (L) to form three circular ribonucleoproteins, which are surrounded by a lipoprotein envelope containing two glycoproteins, G1 and G2. The L RNA segment encodes the RNA polymerase, while the M segment encodes the two spike proteins. The two spike proteins are made from a precursor that is cleaved in the endoplasmic reticulum. The small RNA segment (S segment) gives rise to the nucleocapsid protein, and in members of the *Bunyavirus*, *Phlebovirus*, and *Tospovirus* genera, it also encodes a nonstructural protein termed NS_s (6, 28). In the *Bunyavirus* genus, N and NS_s are translated from overlapping reading frames in the same, viral complementary mRNA (1). For tospoviruses and phleboviruses, the N and NS_s proteins are encoded in an ambisense fashion; i.e., N is translated from viral complementary sense mRNA, and NS_s is translated from a virus-sense mRNA (10, 12, 20, 29, 30). In addition, a nonstructural protein, NS_m, is encoded by the M segment in members of the *Bunyavirus* genus and the phlebovirus group within the *Phlebovirus* genus (4, 5, 8, 13). The other group of phleboviruses, the uukuvirus group, which was recently reclassified into the *Phlebovirus* genus from having been an independent genus, lacks such a nonstructural NS_m protein (3, 27, 29).

The structural proteins, especially the glycoproteins, have been the subject of extensive studies. However, mainly because of the lack of specific antibodies, only a few reports describing the nonstructural proteins have appeared. The intracellular localization of the NS_s protein of three bunyaviruses has been described. In Vero cells infected with Rift Valley fever (RVF) virus (a phlebovirus), NS_s was found to be phosphorylated and rapidly accumulated in filamentous structures in the nucleus (33, 34). On the other hand, in plant cells infected with tomato spotted wilt virus, NS_s remained entirely in the cytoplasm, either dispersed throughout the cytoplasm or forming filamentous or paracrystalline rodlike cytoplasmic structures, depending on the virus isolate studied (15). The NS_s protein of Karimabad virus (a phlebovirus) was likewise found exclusively in the cytoplasmic fraction of infected cells (31). In the case of Punta Toro virus (a phlebovirus), there is no information on the intracellular location, but minute amounts of NS_s have been claimed to be incorporated into virus particles, specifically associated with the nucleocapsids (22). In conclusion, these data provide few clues about the function of the NS_s protein. The picture is further obscured by the fact that it is absent from cells infected by members of the nairoviruses and hantaviruses.

Here we present data on the NS_s protein of Uukuniemi (UUK) virus, a phlebovirus. This protein, which is 273 residues in size (molecular weight of 32,019) is expressed from a subsegmental virus-sense mRNA transcribed from the S RNA segment (29, 30). We report the expression of NS_s in insect cells by using the heterologous baculovirus expression system and the production of a monospecific rabbit anti-NS_s serum, as well as studies of the time

* Corresponding author. Electronic mail address: rpe@kixtal.cmb.ki.se.

course of expression and the half-life of the protein. Furthermore, data concerning the intracellular localization and the association of NS_s with the 40S small ribosomal subunit both in vivo and in vitro are reported.

MATERIALS AND METHODS

Virus and cells. The origin and preparation of the stock virus from the prototype strain S23 of UUK virus have been described previously (24). The titer of the UUK virus stock was 4×10^8 PFU/ml. The virus was propagated in BHK21 cells, clone 13, grown in Eagle's minimal essential medium supplemented with 0.2% bovine serum albumin (BSA), 20 mM HEPES (*N*-2-hydroxyethylpiperizine-*N'*-2-ethanesulfonic acid [pH 7.4]), and antibiotics (24). Cells were infected with a multiplicity of infection of about 10 PFU per cell unless otherwise stated.

Autographa californica nuclear polyhedrosis virus (39) and recombinant baculoviruses were grown in *Spodoptera frugiperda* cells (Sf9 cells). The titers of the virus stocks were in the range of 10^8 to 2×10^8 PFU/ml. The cells were grown in plastic, cell culture flasks at 28°C in TMN-FH medium (Grace's medium supplemented with yeastolate and lactalbumin hydrolysate) containing 10% fetal calf serum (35) and antibiotics. Cells were infected at a multiplicity of infection of 10 PFU per cell.

Construction of recombinant plasmids. Recombinant baculovirus transfer vectors and vectors for in vitro transcription of N and NS_s were constructed as follows. The N gene was excised from the full-length UUK virus S cDNA clone (pUS1037 [29]) as an *Aat*II-*Hin*dII fragment, made blunt ended by using T4 DNA polymerase, and inserted either into the *Sma*I site of the baculovirus transfer vector pVL1392 (19) or into the *Hin*dII site of pGEM-3 (Promega Corp., Madison, Wis.). The NS_s gene was cut out from pUS1037 with *Pst*I and inserted into the *Pst*I site of pVL1392 or pGEM-3. The recombinant baculovirus transfer vectors were termed pVL-UUK/N and pVL-UUK/NS_s, respectively, and the corresponding pGEM-3-based recombinants were termed pGEM-3N and pGEM-3NSB1 or pGEM-3NSB4. In pGEM-3N and pGEM-3NSB4, the N gene and NS_s gene, respectively, were under the control of the T7 promoter, whereas in pGEM-3NSB1, which was used for the in vitro translation experiments, the NS_s gene was inserted downstream of the SP6 promoter.

Generation of recombinant baculoviruses. Wild-type baculovirus DNA was prepared as described by Summers and Smith for the purification of total DNA from infected cells (35). This procedure gives a crude preparation in which approximately 25% of the total nucleic acid content is viral DNA. Cotransfection of plasmid and viral DNA into Sf9 cells was done by the method described by Matsuura et al. (21). At 3 days posttransfection, the virus-containing medium was collected, and recombinant viruses were identified and purified following several consecutive plaque assays performed as described by Brown and Faulkner (2). The recombinant viruses expressing N and NS_s were designated BV-N and BV-NS_s, respectively.

In vitro transcription and translation of N and NS_s. In vitro transcription was performed essentially as described by the manufacturer (Promega Corp.) by using 2 μg of uncut plasmid DNA as template. During the first 15 min of incubation, m^7GpppG was added to a concentration equal to that of the other nucleotide triphosphates (ATP, CTP, and TTP), but GTP was omitted at this stage. GTP was subsequently added to half the concentration of the other nucleoside triphosphates, and incubation was continued for another 60 min.

Reticulocyte lysate for in vitro translation was prepared essentially as previously described (14, 23). To 46 μl of lysate, 2 to 3 μg of RNA, 50 μCi of [^{35}S]methionine (SJ1515, >1,000 Ci/mmol; Amersham Sweden AB, Solna, Sweden), 20 U of RNasin (Promega), and 10 U of Trasylol (Bayer, Leverkusen, Germany) were added, and the lysate was incubated for 60 min at 37°C. The proteins were then immunoprecipitated as described below, or the lysate was used directly for the sucrose gradient experiments.

Metabolic labeling and preparation of cytoplasmic extracts. BHK21 or Sf9 cells, growing as monolayers in culture dishes or flasks, were infected with UUK virus or baculoviruses, respectively, at a multiplicity of infection of 10 PFU per cell. In the case of metabolic labeling with [^{35}S]methionine, cells were washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 [pH 7.4]) (PBS) 1 h prior to the addition of labeled methionine and methionine-free medium was added. [^{35}S]methionine was then added at a concentration of 100 μCi/ml in the same medium. At various times, cells were washed with ice-cold PBS and subsequently lysed in isotonic lysis buffer (120 mM NaCl, 4 mM $MgCl_2$, 30 mM Tris-HCl [pH 7.5]) containing 1% Nonidet P-40 and 100 U of Trasylol (Bayer) per ml. The cells were then scraped off the plates with a rubber policeman, and nuclei and cell debris were pelleted for 5 min in a microcentrifuge. Prior to further use, the pellet was solubilized by being boiled in 1% sodium dodecyl sulfate (SDS) for 3 min. Furthermore, before the nuclei of Sf9 cells infected with wild-type baculovirus were pelleted, the inclusion bodies were disrupted by treating the whole-cell lysate with NaOH at a final concentration of 100 mM.

For the time course experiment, cells were incubated in methionine-free medium 1 h prior to being labeled, labeled for 1 h with [^{35}S]methionine, and lysed as described above. In the pulse-chase experiment, cells were infected with UUK virus, incubated with methionine-free medium at 12 h postinfection (p.i.) for 1 h, and subsequently labeled with [^{35}S]methionine for 30 min. The labeled medium was then removed and replaced by medium containing a 100-fold excess of unlabeled methionine. At various times, the cells were lysed as described above. Protein concentrations of the cell lysates were determined by the method of Hartree (11), and lysate volumes from each fraction corresponding to equal amounts of total protein content were finally subjected to immunoprecipitation.

Immunoprecipitations. Prior to immunoprecipitation, the samples were boiled for 2 min in 1% SDS. The volume was then increased 10-fold by using isotonic lysis buffer, antiserum was added, and the mixture was incubated for 2 h at 4°C. The immune complexes were recovered by binding to protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) for 1 h at 4°C and washed first with NET buffer (400 mM NaCl, 5 mM EDTA, 5 mM Tris-HCl [pH 8.0], 100 U of Trasylol per ml), and then with 10 mM Tris-HCl (pH 8.0). The precipitated proteins were released by being boiled for 2 min in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (100 mM Tris-HCl [pH 8.8], 0.01% bromophenol blue, 12% sucrose, 3.6% SDS) in the presence or absence of DL-dithiothreitol (16 mM), and the reduced samples were finally alkylated with iodoacetamide (60 mM) prior to application onto the gel.

Preparation of antiserum against NS_s. Infection of Sf9 cells (10^7 to 4×10^7 cells) with the recombinant baculovirus BV-NS_s and preparation of cytoplasmic cell extracts were

done as described above. The nuclear fraction of the cells together with traces of radioactively labeled baculovirus-derived NS$_s$ was loaded onto an SDS-PAGE gel. After autoradiography, the NS$_s$ band was cut out, minced, and electroeluted in an electroblotting apparatus (ISCO, Inc., Lincoln, Nebr.) in 10 mM NH_4HCO_3 (pH 8.6). The concentration of the electroeluted protein was determined by densitometry, and 100 to 200 μg of protein was mixed with Freund's complete adjuvant and injected subcutanously into two rabbits. The rabbits were booster injected four times with similar amounts of NS$_s$ in Freund's incomplete adjuvant at 2- to 4-week intervals, and serum was collected 9 to 14 days after each booster.

Affinity purification of anti-NS$_s$ antibodies. Recombinant baculovirus-derived NS$_s$ was separated by SDS-PAGE and transferred by electroblotting to a nitrocellulose filter (Schleicher & Schuell, Dassel, Germany) (see below). The NS$_s$ protein band was identified after being stained with the Ponceau reagent. The cutout filter pieces were subsequently treated for 2 h at room temperature in a blocking reagent (10% fetal calf serum–1% BSA–0.02% sodium azide in PBS) and then incubated overnight, at room temperature, with anti-NS$_s$ serum diluted 1:10 in PBS. After extensive washes on ice with PBS and once with 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–5 mM EDTA, the antibodies were eluted in 0.2 M glycine-HCl (pH 2.8)–0.01% BSA. The eluate was mixed with 0.5 M Na_2PO_4 at a ratio of 5:1, and the filter strips were further washed twice with PBS. Finally, the antibodies were dialyzed against PBS and concentrated in a Minicon microconcentrator (Amicon, Division of W. R. Grace & Co., Danvers, Mass.).

Immunoblotting. Proteins were separated on SDS-polyacrylamide gels (10 to 15% polyacrylamide gradient). Following electrophoresis, the proteins were transferred to nitrocellulose filters (Schleicher & Schuell) by electroblotting (36). The filters were preincubated for a minimum of 2 h at 4°C in blocking buffer I (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5% [wt/vol] skim milk, 1% Tween 20), which subsequently was replaced by antisera diluted 1:100 in blocking buffer I and incubated for 2 h at 37°C. The filters were then washed in blocking buffer II (blocking buffer I containing only 0.1% Tween 20) and incubated with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Promega) diluted in blocking buffer II for 1 h at 37°C. The filters were washed again in blocking buffer II and finally in TSM buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$), and the protein bands were visualized by incubation of the filters with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (Promega).

Transfection of BHK cells. Cells growing in monolayers on 35-mm-diameter plates were infected with recombinant vaccinia virus vTF7-3, which expresses bacteriophage T7 RNA polymerase (7). Forty-five minutes later, the cells were washed twice with transfection medium (Optimem [Life Technologies Ltd., Paisley, Scotland] supplemented with glutamine and antibiotics) and then with 1 ml of transfection medium containing 10 μl of Lipofectin (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Md.), and 1 μg of plasmid DNA was added. Six hours later, the cells were permeabilized, fixed, and subjected to immunofluorescence as described below.

Immunofluorescence. For immunofluorescence, BHK21 cells were grown to subconfluency on coverslips (22 by 22 mm), infected either with UUK virus (multiplicity of infection of approximately 20) for 17 h or with recombinant vaccinia virus expressing T7 RNA polymerase, and in the latter case subsequently transfected as described above. Infected cells were fixed in 3% paraformaldehyde in PBS for 15 min at room temperature. After an additional incubation with 10 mM glycine in PBS for 15 min, the cells were permeabilized with 0.1% Triton X-100 for 30 min. The cells were then stained either with affinity-purified polyclonal anti-NS$_s$ antibody (described above) or with polyclonal anti-N antibodies. Bound antibodies were visualized by incubating the cells with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibodies (Sigma Chemical Co., St. Louis, Mo.).

Sucrose gradient fractionation. Radioactively labeled and freshly prepared lysates from infected or uninfected cells, made as described above, and in vitro translation lysates were loaded onto 10 to 30% linear sucrose gradients in TNE buffer (30 mM Tris-HCl [pH 7.5], 120 mM NaCl, 5 mM EDTA). Centrifugation was done for 6 h at 40,000 rpm in an SW41 rotor, at 4°C, and 0.5-ml fractions were collected from the bottom of the gradient. The pellets were resuspended in 0.5 ml of TNE buffer containing 1% SDS. Thirty microliters of each fraction, including the pellet, was subsequently analyzed by SDS-PAGE without immunoprecipitation.

In some experiments, the lysates were treated with RNase A at a concentration of 30 μg/ml for 30 min at room temperature prior to being loaded onto the gradients, and in other cases, the ionic strength of the lysate was raised by the addition of NaCl to the levels indicated in the Results.

RESULTS

Expression of NS$_s$ and N in Sf9 cells by recombinant baculoviruses. For morphological and biochemical studies of the NS$_s$ protein, antibodies directed against NS$_s$ were produced. Since only minute amounts of NS$_s$ are produced in UUK virus-infected cells (37), the baculovirus expression system was chosen to obtain quantities of protein sufficient for immunization. Parallel to the expression of the NS$_s$ protein, the N protein was also expressed similarly. Prior to the transfer of the NS$_s$ and N genes to the baculovirus transfer vector, pVL1392, the gene fragments were inserted into plasmid pGEM3 and transcribed and translated in vitro. Products with sizes similar to those of authentic N and NS$_s$ obtained from UUK virus-infected cells were observed for the respective constructs (data not shown). The gene fragments were subsequently transferred to pVL1392, and recombinant baculoviruses were created by homologous recombination (35). Recombinant viruses expressing both N and NS$_s$ were obtained (Fig. 1). The size of the [^{35}S]methionine-labeled N protein corresponded to that of the authentic UUK virus-derived N protein. However, the NS$_s$ recombinant sometimes gave rise to two protein bands (Fig. 1, lane 6). The slower one migrated with the same mobility as the UUK virus-derived protein. Further analyses indicated that the faster form corresponds to fully oxidized NS$_s$, while the slower one corresponds to reduced NS$_s$. For some unknown reason, NS$_s$ recovered from the pellet was very difficult to reduce.

The intracellular distribution of N and NS$_s$ in the Sf9 cells did not reflect the situation in UUK virus-infected cells, in which both proteins are found in a soluble cytoplasmic form (see below). Instead, metabolically labeled N and NS$_s$ were found both in the nuclear fraction (pellet) and in the cytoplasmic fractions of infected Sf9 cells (Fig. 1, lanes 4 to 7). Analysis of the total protein content in the nuclear and cytoplasmic fractions on Coomassie blue-stained SDS-polyacrylamide gels showed that the bulk of both N and NS$_s$

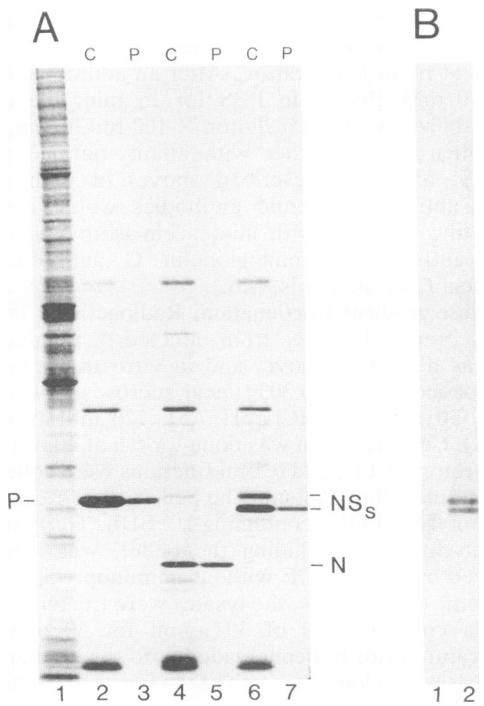

FIG. 1. Expression of NS_s and N in Sf9 insect cells by using recombinant baculoviruses. (A) Infected or uninfected Sf9 cells were labeled at 48 h after infection with [^{35}S]methionine for 1 h, and cytoplasmic and nuclear extracts were prepared as outlined in Materials and Methods. Lane 1, whole-cell extract from uninfected Sf9 cells; lanes 2 through 7, cytoplasmic (C) and nuclear (P) fractions from cells infected with wild-type baculovirus (lanes 2 and 3), BV-N, expressing the N protein, (lanes 4 and 5) or BV-NS_s, expressing the NS_s protein, (lanes 6 and 7). (B) Western blot analysis of cell lysates from uninfected Sf9 cells (lane 1) or BV-NS_s-infected cells (lane 2) by using anti-NS_s serum. P, position of the polyhedrin protein.

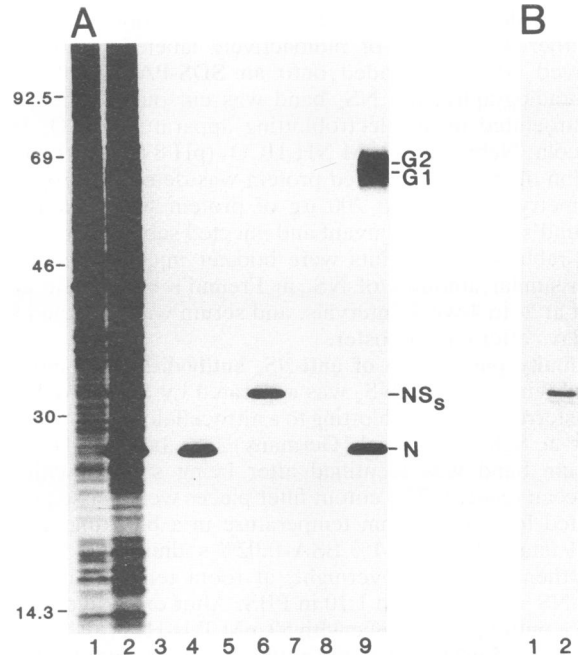

FIG. 2. Immunoprecipitations of NS_s and N from lysates of UUK virus-infected BHK21 cells. (A) Uninfected cells (lanes 1, 7, and 8) or cells infected for 11 h with UUK virus (lanes 3 to 6) were metabolically labeled for 1 h and lysed, and the soluble cytoplasmic extract was subjected to immunoprecipitation with either anti-N (lane 4), anti-NS_s (lane 6), or the corresponding preimmune sera (lanes 3 and 5). Immunoprecipitations from uninfected cells by using anti-N (lane 7) or anti-NS_s (lane 8) served as further controls. Lane 9, purified [^{35}S]methionine-labeled UUK virus. The positions of G1, G2, N, and NS_s are indicated. (B) Western blot analysis of cell lysates from uninfected (lane 1) or UUK virus-infected (lane 2) BHK21 cells by using anti-NS_s serum.

could be found in the low-speed pellets of the cell lysates. The N protein could almost quantitatively be brought into solution by treatment with 4 M urea, whereas NS_s was insoluble even in 8 M urea.

The level of expression of N was somewhat higher than that of NS_s and was only 1/3 to 1/2 of the level of polyhedrin expressed by the wild-type baculovirus. In a typical experiment, about 10 mg of NS_s and 30 to 40 mg of N were obtained from 10^9 cells. There was only a minor difference in the amount of protein produced in cells infected for 48 or 72 h.

Preparation of antiserum against baculovirus-expressed NS_s. The NS_s protein constituted 10 to 25% of the total protein content of the low-speed pellet of recombinant virus-infected cell lysates as judged from Coomassie-stained SDS-polyacrylamide gels. Since a pure protein preparation was needed for immunization and NS_s was insoluble even in 8 M urea, NS_s was isolated from preparative SDS-polyacrylamide gels. The two presumptive NS_s bands expressed by the recombinant baculovirus, BV-NS_s, were identified by autoradiography, together cut out from the gel, and electroeluted. The electroeluted material was then used for immunization of rabbits.

After several boosters, an antiserum was obtained that specifically immunoprecipitated NS_s from lysates of UUK virus-infected BHK21 cells (Fig. 2, lane 6). No proteins were immunoprecipitated from uninfected cell lysates (Fig. 2, lane 8). The antiserum was further shown to be monospecific by immunoblotting of UUK virus-infected BHK21 cell lysates (Fig. 2B, lane 2). However, when lysates of BV-NS_s-infected Sf9 cells were studied by Western blotting (immunoblotting), two bands were regularly observed (Fig. 1B, lane 2). Again, the upper band corresponded to the NS_s band observed in UUK virus-infected BHK21 cells. As mentioned above, the two bands represent the reduced and oxidized forms of NS_s.

The specificity of the anti-N serum was also studied in parallel and was shown to be monospecific by the same criteria used for the NS_s serum (Fig. 2, lanes 4 and 7).

Kinetics of expression and half-life of NS_s compared with those of N. The time course of expression of N and NS_s in chicken embryo fibroblasts has previously been studied by Ulmanen et al. (37). The N protein was shown to first appear at 6 h p.i., whereas the NS_s protein was evident some 2 h later. With the aid of the antisera directed against the two proteins, we wanted to repeat these experiments with BHK21 cells, the cell line currently used to propagate UUK virus. Infected cells were labeled for 1 h at the indicated times (Fig. 3), and N and NS_s were recovered from the cell lysates by immunoprecipitation and analyzed by SDS-PAGE. By this assay, expression of N was detected as early as 4 h p.i., whereas appreciable amounts of NS_s could not be detected until 8 h p.i. (Fig. 3A).

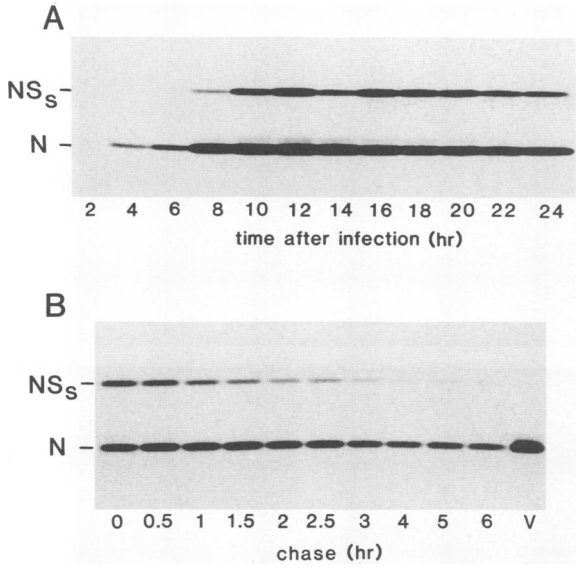

FIG. 3. Time course of expression and stability of the NS$_s$ and N proteins. (A) UUK virus-infected BHK21 cells were labeled with [^{35}S]methionine for 1 h at different times p.i. and subsequently lysed at the indicated times. The NS$_s$ and N proteins were immunoprecipitated and analyzed by SDS-PAGE. (B) UUK virus-infected cells were labeled with [^{35}S]methionine for 30 min at 12 h p.i., chased in the presence of an excess of unlabeled methionine for the indicated periods of time, and analyzed by SDS-PAGE following immunoprecipitation. V, virion-derived N protein marker.

The stability of NS$_s$ was also studied and compared with that of the N protein. To this end, cells were radioactively labeled for 30 min, chased for various times in the presence of unlabeled methionine, and analyzed by SDS-PAGE. The gel shown in Fig. 3B was densitometrically scanned, and the half-life of NS$_s$ was estimated to be approximately 1.5 to 2 h. The N protein, in contrast, was much more stable, with a half-life of several hours. Since cycloheximide was not used during the chase, virus particle formation continued. Therefore, a portion of the N protein was lost from cells in virus particles.

Intracellular localization of NS$_s$. To obtain information concerning the site of action of NS$_s$, immunofluorescence was performed with UUK virus-infected BHK21 cells fixed with paraformaldehyde at 17 h p.i. For this purpose, an affinity-purified preparation of anti-NS$_s$ was used, since the crude antisera suitable for immunoprecipitation and Western blotting were shown to give a high level of unspecific background staining. With the affinity-purified anti-NS$_s$ antibody, NS$_s$ displayed a punctate distribution throughout the cytoplasm, with no indication of nuclear staining and also no particular intracellular sites of accumulation (Fig. 4A and B). The staining was specific as evidenced by the total absence of fluorescence in uninfected cells (Fig. 4C).

The intracellular localization of NS$_s$ as well as of N was further studied with BHK21 cells expressing the proteins with the aid of a heterologous expression system and hence in the absence of other UUK viral proteins. For this purpose, cells were infected with a recombinant vaccinia virus, vTF7-3, expressing T7 RNA polymerase (7) and subsequently transfected with plasmids having the NS$_s$ or N gene inserted downstream of the T7 Φ10 promoter. Cells expressing NS$_s$ showed a punctate cytoplasmic immunofluorescence pattern (Fig. 4D), very similar to the distribution of NS$_s$ in UUK virus-infected cells.

In UUK virus-infected cells, the N protein also showed disperse cytoplasmic staining, but in addition, an accumulation in the Golgi region was obvious at later times during infection. This accumulation is thought to be due to the association of the nucleocapsids with the viral spike proteins, G1 and G2, which reside in the Golgi complex (17, 18). In the heterologous expression system, only punctate cytoplasmic staining was seen for the N protein, without accumulation in the Golgi region (data not shown). For some cells expressing either NS$_s$ or N, a perinuclear staining could be observed. However, this staining did not resemble Golgi staining but probably represented unspecific staining of large vaccinia virus-induced inclusions also seen in mock-transfected cells.

Cosedimentation of NS$_s$ with the 40S ribosomal subunit. The disperse cytoplasmic staining of NS$_s$ in infected cells late in the infection cycle suggested that the protein does not participate directly in virus budding in the Golgi complex. Also, repeated attempts to identify NS$_s$ in [^{35}S]methionine-labeled virus preparations or nucleocapsids have failed (data not shown), confirming the nonstructural nature of NS$_s$ suggested in an earlier study (37). One approach for gaining insight into the function of the NS$_s$ protein would be to study the interaction of NS$_s$ with other viral or cellular macromolecules. One possibility could be that NS$_s$ would transiently interact with the viral ribonucleoproteins during, for example, their assembly. To study this possibility, [^{35}S]methionine-labeled lysates from UUK virus-infected BHK21 cells were fractionated on 10 to 30% sucrose gradients. In such gradients, NS$_s$ was found to sediment into a discrete peak. No other major labeled proteins cosedimented with NS$_s$ (Fig. 5A). The N protein, which under these conditions is bound to the three RNA species as nucleoproteins, was recovered throughout the gradient. The sedimentation of NS$_s$ did not follow that of the N protein, suggesting that NS$_s$ is not associated with the nucleoproteins. In order to determine the sedimentation coefficient of NS$_s$, the positions of the 60S and 40S ribosomal subunits were determined by analyzing the RNA content of each fraction. The NS$_s$ protein was found in the same fractions as was the 18S rRNA and was, hence, cosedimenting with the 40S small ribosomal subunit. In some experiments, a faint band of the size of NS$_s$ could also be seen in the fractions containing the 60S ribosomal subunit. Very little free NS$_s$ protein was recovered from the top of the gradient, as shown by immunoprecipitation of individual fractions with the anti-NS$_s$ serum (data not shown).

To verify the involvement of the 40S subunit in binding NS$_s$, the lysate was treated with RNase A prior to application onto the gradient. After such a treatment, NS$_s$ was no longer recovered at the position of the 40S subunit. Instead NS$_s$ was found on top of the gradient (data not shown), further strengthening the conclusion that NS$_s$ is indeed associated with the 40S ribosomal subunit.

When NS$_s$ was expressed by using the T7 vaccinia virus expression system and labeled for 5 min in the presence of [^{35}S]methionine, the bulk of the protein was found to cofractionate with the 40S ribosomal subunit (Fig. 6A). A 30-min chase in the presence of excess unlabeled methionine did not significantly change the distribution of NS$_s$ in the gradient (Fig. 6B). We also studied whether NS$_s$ that had been released from the ribosomes by high-salt treatment could reassociate with the 40S ribosomal subunit. In this case, BHK cells infected with UUK virus were labeled in the

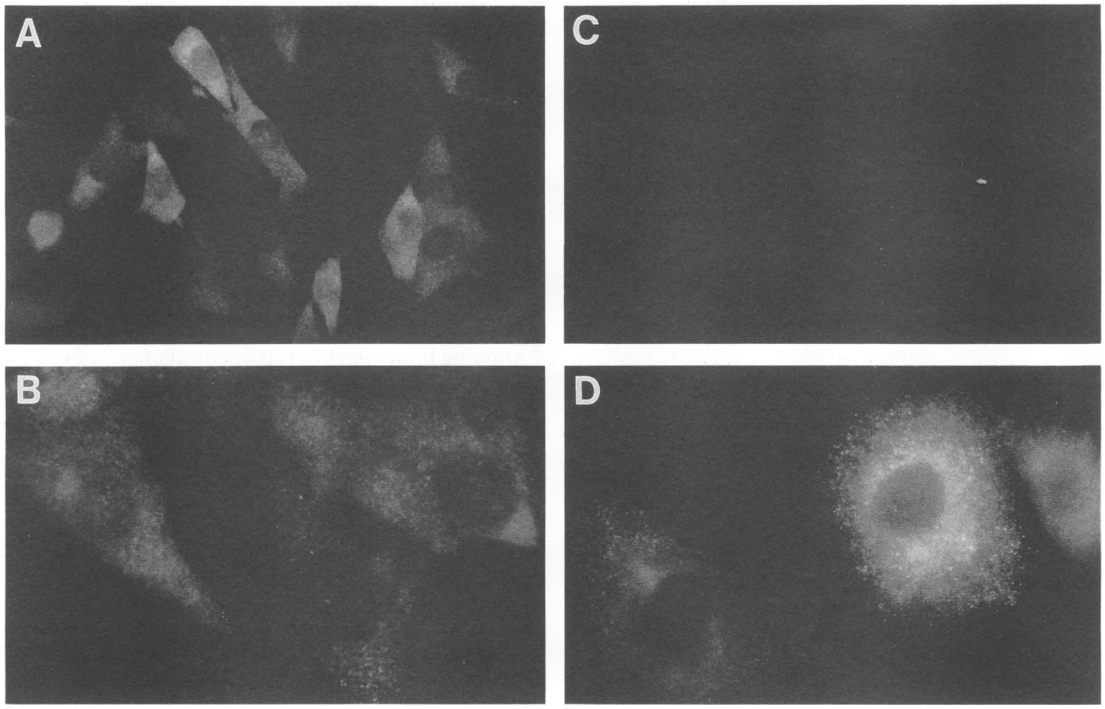

FIG. 4. Immunolocalization of NS_s in UUK virus-infected and transfected BHK21 cells. UUK virus-infected cells were indirectly stained at 17 h p.i. by using an affinity-purified anti-NS_s antibody and fluorescein isothiocyanate-conjugated secondary antibody. In low (A)- and high (B)-magnification micrographs, diffuse, punctate cytoplasmic staining is evident, while uninfected cells (C) show no staining. Immunolocalization was also performed with BHK21 cells programmed to express NS_s (D) by using the T7 recombinant vaccinia virus expression system described in Materials and Methods. A staining pattern very similar to the one seen for UUK virus-infected cells is evident.

presence of [^{35}S]methionine, and a cytoplasmic extract was prepared by homogenizing the cells in a hypotonic buffer by using a tightly fitting Dounce homogenizer. The homogenate was subjected to NaCl at a final concentration of 1.5 M and finally dialyzed against isotonic buffer. The lysate was then mixed with fresh, unlabeled lysate from uninfected BHK cells, incubated for 30 min on ice, and analyzed on a sucrose gradient as described before. After the high-salt treatment, NS_s was found on top of the gradient, whereas it cofractioned with both the 40S and 60S ribosomal subunits following dialysis. This result indicated that the binding to the 40S ribosomal subunit is specific and closely associated with the translation of NS_s, whereas the binding to the 60S subunit may be unspecific (data not shown). We have also found that NS_s associates with the 40S ribosomal subunit in Sf9 cells infected with BV-NS_s.

We extended the in vivo study by analyzing the fate of NS_s translated in vitro in a rabbit reticulocyte lysate. In vitro-made NS_s was again found to cofractionate with the 40S ribosomal subunit (Fig. 7), while the N protein, cotranslated together with NS_s, was recovered from the top of the gradient. As for the in vivo situation, in vitro-made NS_s also shifted to the top of the gradient after RNase A treatment.

We also studied the resistance of the interaction to increasing ionic strength. In these experiments, the salt concentration of either cell lysates or lysates of in vitro-translated NS_s was increased by the addition of NaCl prior to application of the lysates onto the sucrose gradient. Final salt concentrations were 300 mM, 600 mM, 1 M, and 1.5 M NaCl. No effect was seen with the two lowest concentrations of salt. At 1 M NaCl, a partial disruption of the interaction could be seen, causing a portion of the NS_s protein to sediment in lighter fractions of the gradient. At still higher salt concentrations (1.5 M NaCl), the release of NS_s was complete, by using both in vivo- and in vitro-derived NS_s (results not shown). Analyses of the position of 28S and 18S RNAs indicated that the treatment did not disrupt the ribosomal subunits, even at the highest salt concentration.

DISCUSSION

We have initiated the characterization of the NS_s protein of UUK virus with the ultimate goal of understanding the function of the protein. As an aid in such studies, we have produced a monospecific antibody against baculovirus-expressed NS_s. Our results so far can be summarized as follows. NS_s was dispersed throughout the cytoplasm of BHK21 cells, in which it was firmly associated with the small 40S ribosomal subunit. In vitro-translated NS_s or NS_s expressed in BHK21 cells or Sf9 insect cells from cloned cDNA likewise was associated with the 40S subunit. NS_s was not found in the nucleus or in extracellular virions. No interaction with intracellular nucleocapsids could be demonstrated, nor was any evidence for phosphorylation obtained. Finally, detectable levels of NS_s appeared a few hours later than those of N, and the half-life of NS_s is rather short (1.5 to 2 h) compared with that of the N protein.

The localization of NS_s to the cytoplasm was based on the fact that metabolically labeled NS_s was found only in the cytoplasmic fraction associated with the 40S ribosomal subunit and that immunofluorescence revealed punctate, dispersed, exclusively cytoplasmic staining. A similar distribution was also observed when NS_s was expressed alone by using the T7 vaccinia virus system. The granular, punctate

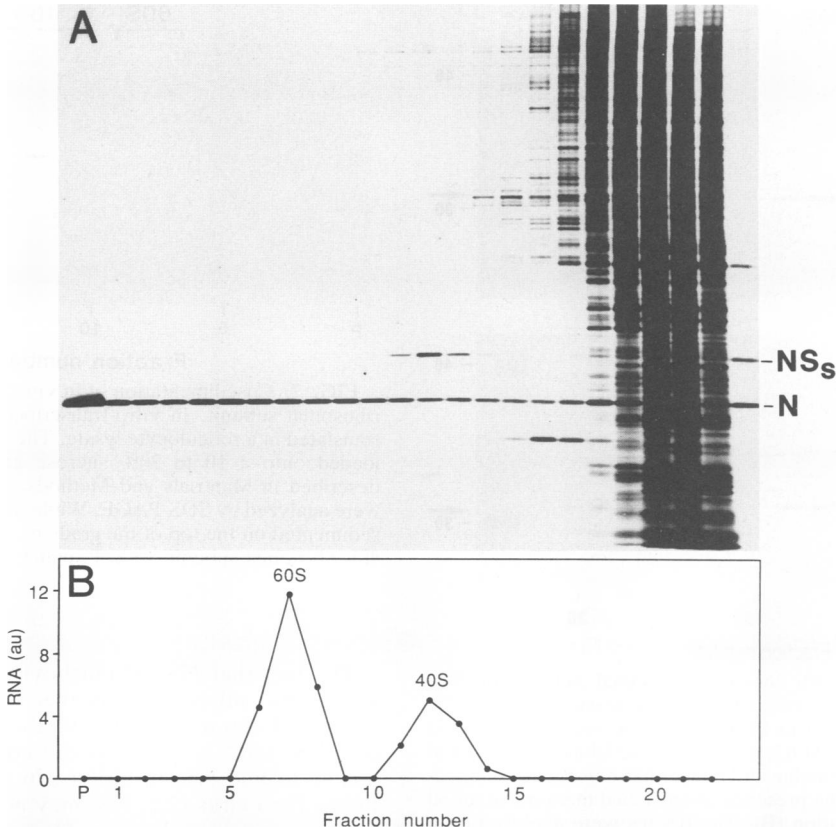

FIG. 5. Cosedimentation of NS$_s$ with the smaller 40S ribosomal subunit. (A) A cytoplasmic extract prepared from [^{35}S]methionine-labeled, UUK virus-infected BHK21 cells was fractionated on a 10 to 30% sucrose gradient, as described in Materials and Methods. Aliquots from each were directly analyzed by SDS-PAGE. The majority of the NS$_s$ protein was found to cosediment with the 40S ribosomal subunit, the position of which was determined by analysis of the RNA content in each fraction by agarose gel electrophoresis. The N protein, which is present in both a free form and a ribonucleoprotein-bound form, was distributed throughout the gradient. (B) Recovery of 28S (60S peak) and 18S (40S peak) RNA. P, pellet; au, arbitrary absorbance units.

immunostaining pattern is in conformity with the notion that most NS$_s$ is associated with ribosomes. NS$_s$ of RVF virus has been found by immunofluorescence to accumulate in filamentous inclusions in the nucleus, and metabolically labeled NS$_s$ was recovered in the nuclear fraction (34). It should be noted that a hyperimmune sheep serum or mouse ascitic fluid was used in the previous studies, while we used a monospecific affinity-purified antibody. In contrast to RVF virus, metabolically labeled NS$_s$ of Karimabad virus, another phlebovirus, was found exclusively in the cytoplasmic fraction (31). Similarly, NS$_s$ of tomato spotted wilt virus was detected by immunogold labeling dispersed throughout the cytoplasm or associated with cytoplasmic fibers or filaments (15). In UUK virus-infected cells, no inclusions or filaments were evident by immunofluorescence staining. The basis for the different intracellular localizations of NS$_s$ of different viruses remains unclear but may relate to the virus strain, the level of NS$_s$ expression, the host cell, or the nature of the antisera used.

The interaction of NS$_s$ with the 40S subunit occurred shortly after synthesis and was surprisingly resistant to high concentrations of salt. Only after treatment with NaCl at concentrations above 1 M was the NS$_s$ protein released. The capsid protein C of Semliki Forest virus (an alphavirus) has also been found to associate strongly with ribosomes, in this case, the 60S subunit, immediately after synthesis (38). It is then transferred to the genomic 42S RNA to form the nucleocapsid (32). The association of the C protein to the 60S subunit was sensitive to high concentrations of salt, 60 to 70% of C being released by 0.8 M KCl (38). Cross-linking experiments have shown that the C protein interacts with the 28S rRNA (26). Association of virus-specific proteins to ribosomes in a high-salt-sensitive manner has also been reported for other viruses, such as picornaviruses and influenza viruses (38), but the significance of these interactions has not been elucidated.

The NS$_s$ of RVF virus has been reported to be phosphorylated (34). We were unable to find evidence for phosphorylation of NS$_s$ of UUK virus by metabolical labeling with $^{32}P_i$. The double band occasionally seen on SDS-polyacrylamide gels with NS$_s$ isolated from infected BHK21 cells and regularly seen with NS$_s$ from Sf9 insect cells was found to be due to differences in the degree of disulfide bond formation probably induced after disruption of the cells. Treatment of NS$_s$ with alkaline phosphatase did not alter its mobility, further supporting the conclusion that NS$_s$ is not phosphorylated.

NS$_s$ has been claimed to be incorporated into Punta Toro virus virions and to be associated with nucleocapsids (22). We were unable to detect NS$_s$ in virions, nor were any traces of NS$_s$ found in purified tomato spotted wilt virus (15). However, if NS$_s$ is present in, for example, only one copy per ribonucleoprotein segment, its detection would be ex-

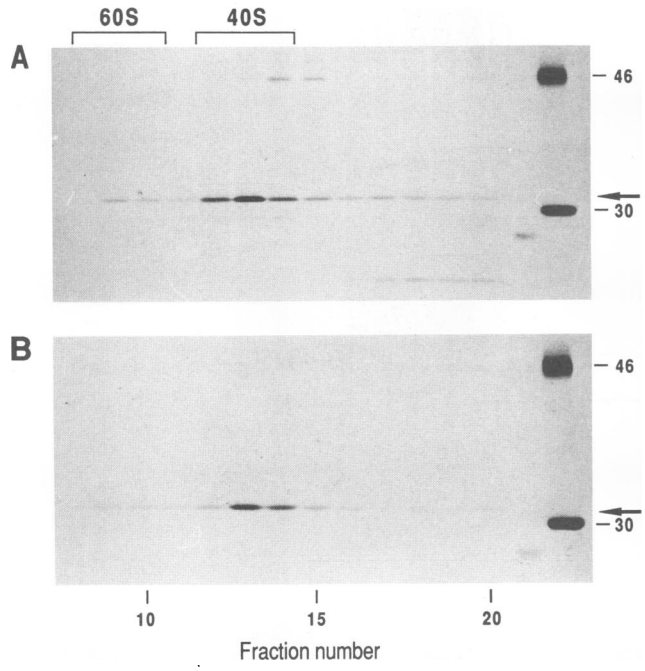

FIG. 6. Association with the 40S ribosomal subunit of NS_s expressed by using the T7 vaccinia virus expression system. The NS_s protein was expressed in BHK21 cells by using the vaccinia virus expression system. At 6 h p.i., cells were labeled for 5 min in the presence of [^{35}S]methionine and then either homogenized immediately (A) or chased in the presence of unlabeled methionine for 30 min prior to homogenization (B). The lysates were analyzed on a sucrose gradient and subjected to immunoprecipitation with the anti-NS_s serum prior to SDS-PAGE. The arrows indicate the position of NS_s.

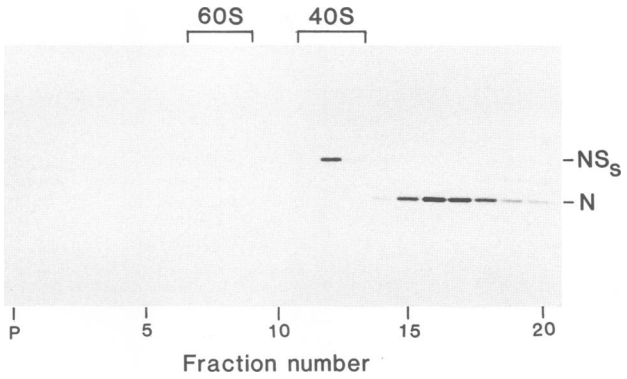

FIG. 7. Cosedimentation of in vitro translated NS_s with the 40S ribosomal subunit. In vitro-transcribed NS_s and N mRNAs were translated in a reticulocyte lysate. The translation mixture was then loaded onto a 10 to 30% sucrose gradient and fractionated as described in Materials and Methods. Aliquots from each fraction were analyzed by SDS-PAGE. While the N protein used as a control sedimented on the top of the gradient, NS_s was recovered from the 40S ribosomal subunit peak. P, pellet.

tremely difficult. Since NS_s is associated with ribosomes, even minor contamination with host membranes or ribosomes could give the false impression that NS_s is associated with purified virions.

The function of NS_s is unknown for any of the bunyaviruses. The hantaviruses and nairoviruses apparently do not code for any NS_s protein (6, 28). In the case of hantaviruses, the complete genome has been sequenced, and no open reading frame that could give rise to a protein corresponding to NS_s is present. The S segment of hantaviruses and nairoviruses has the same size as that of the UUK virus (about 1,700 nucleotides), but the N protein is substantially larger in the two former viruses (430 to 440 amino acids) compared with that in the UUK virus (273 residues) and other phleboviruses or bunyaviruses (6, 28, 29). Thus, there is a possibility that the function(s) of NS_s is contained within the N protein of hantaviruses and nairoviruses. Alternatively, the NS_s function may have been incorporated into the large L protein. The NS_s protein is the most divergent of all bunyavirus proteins. Although the L, G1, G2, and N proteins display various degrees of sequence homologies between the UUK virus and other phleboviruses, the NS_s proteins show a very low degree of homology. Interestingly, a mutant RVF virus that has a 600-nucleotide deletion in the coding sequence of NS_s in the S segment replicates to wild-type virus levels in Vero and mosquito cells but is defective in human lung diploid, MRC-5, cells (27a). In light of some of these results, it is possible that the function(s) supplied by NS_s is dispensable.

The fact that NS_s of phleboviruses is translated from a virus-sense mRNA means that the S segment has to be replicated into a full-length viral-complementary strand before NS_s mRNA synthesis can commence. Since we were unable to find NS_s in virions, in contrast to the report on Punta Toro virus (22), NS_s may not be involved in primary transcription or replication. However, in a previous report, we noted that UUK virions contain both full-length virus-sense and viral-complementary sense S RNAs in a molar ratio of roughly 10:1 (29). This finding has not yet been confirmed for any other bunyavirus, and its significance therefore remains unclear; however, it suggests that the NS_s mRNA can be transcribed concomitantly with the N mRNA without the need for prior replication.

What possible functions could NS_s then have? In the absence of sufficient information, one can only speculate. Its association with the ribosomes per se does not offer a plausible hypothesis as to the function. NS_s could be involved in the regulation of transcription or replication. One possibility is that it could be involved in the cap snatching shown to occur in the cytoplasm of bunyavirus- and phlebovirus-infected cells. Similar to those of influenza virus mRNAs, short stretches of host-derived sequences have been demonstrated at the 5′ ends of mRNAs of several bunyaviruses (6, 30). NS_s could serve one of the functions carried out by one of the three polymerase subunits of influenza virus (16). The fact that the synthesis of NS_s starts rather late during UUK virus infection suggests that it may be involved in virus assembly. It could, for example, assist in governing the ribonucleoproteins to the Golgi complex or facilitate the packaging of a correct set of ribonucleoproteins into budding particles. However, although G1, G2, and N accumulate in the Golgi complex during infection (17, 18), NS_s did not become concentrated to the site of budding.

It is unlikely that further biochemical characterization of NS_s will shed much light on its function. The most productive approach is likely to be the analysis of the phenotype of conditional mutants mapping to the NS_s gene. Quite a number of temperature-sensitive mutants have been isolated for some bunyaviruses expressing an NS_s protein (25), including UUK virus (9). We are currently exploring the

genotypes and phenotypes of some UUK virus *ts* mutants with the hope of unraveling the function of NS$_S$.

ACKNOWLEDGMENTS

We thank Anita Bergström for excellent help with the cell culturing, Jaakko Saraste for helpful advice concerning the affinity purification of the antibody, Sune Kvist for preparing the rabbit reticulocyte lysate, Max D. Summers and Verne A. Luckow for the pVL1392 transfer vector, Bernard Moss for the recombinant vaccinia virus, vTF7-3, and Ulf Eriksson for valuable comments during the progress of the work.

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