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In Vitro Translation of Uukuniemi Virus-Specific RNAs: Identification of a Nonstructural Protein and a Precursor to the Membrane Glycoproteins

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We isolated the virus-specific RNA species from Uukuniemi virus-infected chicken embryo cells and fractionated them by sucrose gradient centrifugation. In addition to three RNA species cosedimenting with the three viral RNA segments L (29S), M (23S), and S (17S), a fourth major RNA species, sedimenting at about 12S (S2), was found early in the infection. Annealing experiments indicated that the cytoplasmic L and M RNA species consisted of both plus and minus strands, with the plus strands in slight excess. Most of the S1 RNA was of negative polarity, whereas S2 was of positive polarity. The S2 RNA specifically annealed to the virion S RNA segment, indicating that it is transcribed from this segment. In vitro translation of the individual RNA species in micrococcal nuclease-treated cell-free retjculocyte extracts showed that an mRNA cosedimenting with the virion M RNA directed the synthesis of a virus-specific 110,000-dalton polypeptide (p110). This polypeptide could be immunoprecipitated with antiserum prepared against purified virions. When translation was carried out in the presence of dog pancreas microsomes, p110 was absent. Instead, an immunoprecipitable polypeptide band, with a molecular weight of about 70,000 and migrating between the virion surface glycoproteins G1 and G2, was observed. It is thus likely that the glycoproteins are synthesized as a precursor (p110), which during translation is cleaved roughly in the middle to yield G1 and G2. The 12S RNA species directed the synthesis of the nucleocapsid protein and a novel polypeptide with an apparent molecular weight of about 30,000. The latter was not precipitated with antivirion serum and was absent from lysates programmed with the corresponding RNA fraction from a mock-infected extract. Since, in addition, it was not found in purified virions and was present in the cytoplasm of infected cells but not in uninfected cells, it probably represents a nonstructural polypeptide.

The Bunyaviridae family comprises a large and heterogeneous group of arthropod-borne viruses (24). All members so far characterized have certain structural features in common. Thus, they all contain a segmented single-stranded RNA genome; regularly, three species of RNA (L, M, and S) with a negative polarity have been found in purified virions (4, 5, 7, 17, 21, 25). The size of the segments varies from one member to another (17). Fingerprint analysis of ^{32}P-labeled RNA segments have unambiguously shown that each segment contains a unique nucleotide sequence and thus must encode different polypeptides (7, 21). Four structural proteins are found in virions (16, 17). The largest, L, is present in small amounts and is often difficult to detect in purified virions (16). The two glycoproteins, G1 and G2, form the spikes on the viral surface, whereas the smallest, N, is the nucleocapsid protein (16, 23). Again, the size of the polypeptides varies from one virus to another (17).

On the basis of genetic recombination (reassortment of RNA segments) between temperature-sensitive mutants of different viruses within the California encephalitis virus complex, it has been concluded that the M RNA contains the information for the two glycoproteins (11). In vitro translation of snowshoe hare virus-specific RNA isolated from infected cells has indicated that the S RNA segment encodes the nucleocapsid (N) protein (6, 10). By exclusion it is therefore likely that the L RNA encodes the L protein.

Uukuniemi viruses form a distinct serological group within the Bunyaviridae family (24). The prototype of the group, Uukuniemi virus, has been the subject of many detailed molecular analyses. The virion contains three RNA segments, L (molecular weight, ~2.4 ± 0.4 × 10^6),

M (molecular weight, 1.1 ± 0.2 × 10^6), and S (molecular weight, 0.5 ± 0.1 × 10^6), which by RNA fingerprinting have been shown to contain unique nucleotide sequences (21). As is the case in other bunyaviruses, four structural proteins have been recognized: L (molecular weight, 180,000 to 200,000) (Pettersson, unpublished data), G1 (molecular weight, ~75,000), G2 (molecular weight, ~65,000), and N (molecular weight, 25,000) (23, 28). It is therefore clear that one of the RNA segments must encode at least two polypeptides. So far, it has not been known which RNA segment encodes the different proteins. To study this, we have isolated, fractionated, and translated in vitro the virus-specific RNAs from infected cells and found that an mRNA species of the same size as the virion M RNA codes for a precursor to the two glycoproteins and that a 12S mRNA species, which is transcribed from the virion S segment, codes for the N protein and a novel nonstructural (NS) polypeptide.

MATERIALS AND METHODS

Preparation of [^{35}S]methionine-labeled cytoplasmic extracts. The origin and cultivation of chicken embryo cells as well as preparation of stock virus from the prototype strain S23 of Uukuniemi virus have been described previously (22). Virus was labeled with [^{35}S]methionine and purified by sucrose gradient centrifugation as described previously (22, 28).

Secondary cultures of chicken embryo cells grown on 20-cm^2 petri dishes as monolayers were infected with Uukuniemi virus at a multiplicity of about 10 PFU per cell. After a 60-min adsorption period at 37°C the unadsorbed virus was discarded, and the cells were washed once with Hanks salt solution and then maintained in Eagle minimum essential medium supplemented with 0.2% bovine serum albumin. At different times after infection, methionine-free minimum essential medium was added 1 h before labeling of the cells. [^{35}S]methionine (Radiochemical Centre, Amersham, 750 Ci/mmol) in 2 ml of methionine-free minimum essential medium (100 μCi per dish) was added for 1 h. Mock-infected cells were treated and labeled similarly but the virus inoculum was omitted.

After labeling, the cells were washed twice with phosphate-buffered saline, scraped off the plate, and concentrated by centrifugation at 1,200 rpm for 10 min. The cells were suspended in 0.5 ml of RSB-Na buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-hydrochloride [pH 7.4]) and allowed to swell for 15 min at 0°C. The cells were then disrupted by 30 strokes in a Dounce homogenizer, and the nuclei were removed by centrifugation for 5 min at 1,500 rpm. Triton X-100 (1% final) was added to the supernatant, and the nuclei were washed once with RSB-Na containing 1% Triton X-100. The nuclear wash and the supernatant were pooled and used for immunoprecipitation and analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (13).

Isolation of RNA from infected and mock-infected cells. One-liter roller bottles of secondary chicken embryo cells were infected as described above. Mock-infected bottles were treated similarly throughout the infection but the virus inoculum was omitted. At 4.5 h postinfection (p.i.), actinomycin D was added to both sets of cultures (1 μg/ml) and the virus-specific RNA was labeled between 5 and 8 h p.i. with [^{3}H]-uridine (1 mCi per bottle; Amersham, 25 Ci/mmol). For annealing experiments, actinomycin D was added at 1 h, and the RNA was labeled from 1.5 to 8 h p.i. At 8 h p.i., the cells were washed twice with phosphate-buffered saline and 5 ml of trypsin-EDTA was added to each bottle for 5 min at 37°C. After addition of calf serum, the cells were collected by centrifugation and washed twice with ice-cold phosphate-buffered saline and once with RSB-Na. The cells were then resuspended in 7.5 ml of RSB-Na, allowed to swell for 10 min at 0°C, and disrupted by 20 strokes in a tight-fitting Dounce homogenizer. The nuclei were removed by centrifugation, and SDS (2% final concentration) was added to the supernatant (cytoplasmic extract). The RNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol at 0.3 M sodium acetate and −20°C. The RNA precipitate was suspended in 0.5 ml of TNE buffer (140 mM NaCl, 10 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA) containing 0.5% SDS and layered on a 15 to 30% (wt/wt) sucrose gradient made in TNE plus 0.5% SDS. Centrifugation was for 17 h at 25,000 rpm and 23°C in an SW27 rotor. Trichloroacetic acid-precipitable radioactivity was determined from samples of sucrose gradient fractions. Relevant fractions were pooled and the RNA was precipitated twice with ethanol to remove residual SDS, lyophilized, and suspended in double-distilled water immediately before translation in vitro. RNA samples were stored in water at −70°C in aliquots.

Preparation of rabbit reticulocyte lysate and translation in vitro. Reticulocyte lysate was prepared from the blood of anemic rabbits as described by Villa-Komaroff et al. (27) and made mRNA dependent by treatment with micrococcal nuclease by the method of Pelham and Jackson (18). A standard assay mixture (25 μl) contained 10 μl of lysate, 0.5 mM spermidine (Sigma Chemical Co.), 25 μM amino acids (omitting methionine) (BDH), 2 mM dithiothreitol (Calbiochem), 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.6; Sigma), 16 μg of hemin (Eastman Organic Chemicals) per ml, 8 mM creatine phosphate, 140 μg of creatine phosphokinase (Sigma) per ml, 30 μCi of [^{35}S]methionine (Amersham), 120 mM potassium acetate, 0.3 mM magnesium acetate, and 20 μg of calf liver tRNA (Boehringer Mannheim Corp.) per ml. The amount of RNA added was not standardized.

EDTA-stripped dog pancreas microsomes (1, 2) were kindly prepared by B. Dobberstein and obtained from S. Kvist. The concentration of microsomes in the assay mixture was 12.5 optical density units per ml at 280 nm. All translation assays were performed at 28°C and incubated for 2.5 h. The reactions were stopped by adding SDS (1% final concentration), and samples were taken for polyacrylamide gel electrophoresis and immunoprecipitation.

Immunoprecipitation and analysis of proteins by SDS-gel electrophoresis. Immunoprecipitation with Formalin-fixed *Staphylococcus aureus* (Cowan strain I) as the immunoadsorbent for immunoglobulin G was performed as described by Persson et al. (19). The procedure included incubation of diluted cell extract or in vitro reaction mixtures with normal rabbit serum (1 h at 20°C and 1 h at 0°C, respectively) followed by mixing of the bacteria with the samples. Bacteria were collected by centrifugation, and the supernatant was used for immunoprecipitation with either immune or preimmune serum (10 μl per assay). Incubations were as above, and the precipitates were washed twice with phosphate-buffered saline and once with water before adding the gel sample mixture and heating for 2 min at 100°C.

Antiserum against purified Uukuniemi virus was prepared in rabbits by injecting 100 μg of Triton X-100-disrupted virus in Freund complete adjuvant. Three booster injections of 70 μg of virus each were given subcutaneously at 3-week intervals. Rabbits were bled before each injection, and the sera were checked for the presence of antibodies against the major viral structural proteins. Protein samples were analyzed in SDS-polyacrylamide slab gels by the method of Laemmli (13) with a 10% separating and a 5% stacking gel and developed by fluorography by the method of Bonner and Laskey (3).

Hybridization experiments. To analyze the polarity of the cytoplasmic RNA species, the RNAs were recovered from the sucrose gradient pools by ethanol precipitation. Self-annealing and annealing to an excess of virion RNA (5 μg per incubation) were carried out in a volume of 100 μl in sealed glass capillaries for 5 to 7 h at 65°C in the presence of 5× SSC (1× SSC is 0.015 M sodium citrate plus 0.15 M NaCl [pH 7.5]) and 0.05% SDS. Melting of RNA was done at 100°C for 2 min and followed by quenching on ice. RNase resistance was determined as the percentage of acid-precipitable radioactivity recovered after treatment of half of the sample with pancreatic RNase (40 μg/ml, 37°C, 20 min) as compared with the undigested control. Before digestion, the samples were diluted 10-fold to lower the SDS concentration to 0.005% and the salt concentration to 0.2 M NaCl.

RESULTS

In vivo synthesis of virus-specific polypeptides. It was previously shown (20) that Uukuniemi virus does not shut off host cell protein synthesis in chicken embryo cells even late in the infection. Therefore, it was likely that identification of virus-specific proteins against the background of host proteins would be difficult. To get an idea about the level and kinetics of viral protein synthesis, we labeled infected and mock-infected cells for 1 h with [^{35}S]methionine at various times after infection and analyzed the labeled proteins by SDS-gel electrophoresis (Fig. 1). In agreement with previous results, the gel revealed that host cell protein synthesis indeed is not shut off even at 36 h p.i. The only identifiable viral structural protein was

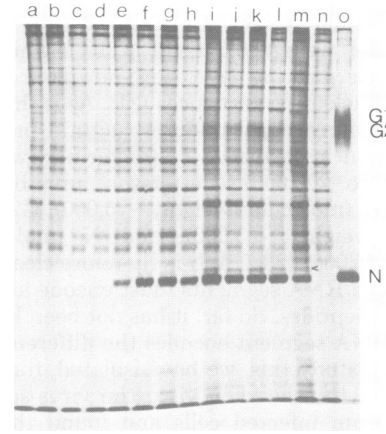

FIG. 1. *Pulse-labeling of proteins in Uukuniemi virus-infected cells at various times after infection. Chicken embryo cells, infected with Uukuniemi virus, were pulse-labeled with [^{35}S]methionine for 1 h at various times after infection. Samples of the cytoplasmic extracts were analyzed on a 10% SDS-polyacrylamide gel. (a and n) Samples prepared from mock-infected cells at the beginning and end of the experiment. (o) [^{35}S]methionine-labeled virion protein markers. (b through m) Infected cells labeled at 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 36 h p.i., respectively. Arrow indicates the position of the tentative NS protein.*

the N protein, which started to appear at about 6 h p.i. (Fig. 1, lane e) and was synthesized throughout the period studied (to 37 h p.i.). Neither the G1 and G2 proteins, which did not separate well in this run (Fig. 1, lane o), nor the L protein can be seen at any time. A polypeptide, migrating slightly slower than the N protein and absent from mock-infected cells (Fig. 1, lanes a and n) can be clearly seen (Fig. 1, lane m, arrow). This protein band is visible from about 8 h p.i. (Fig. 1, lanes f to m).

To reveal the glycoproteins, the viral structural proteins were immunoprecipitated from the extracts with antiserum prepared against whole virions (Fig. 2). Both the G1 and G2 (first visible from about 14 h p.i. with this method) (Fig. 2, lane e) and the N proteins (visible from 10 h p.i.) (Fig. 2, land d) were immunoprecipitated with the antiserum. In addition, some precipitable material of unknown identity was seen at the top of the gel. The protein migrating slightly slower than the N protein (Fig. 1, lane m, arrow) did not immunoprecipitate.

Isolation of RNA from infected and mock-infected cells. According to the results shown in Fig. 1, the N protein is first detected at about 6 h p.i., at a time when very few virions are released from the cells (20). To enrich for virus-specific mRNA, a cytoplasmic extract was there-

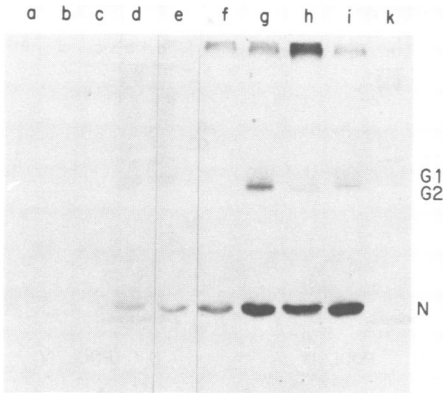

FIG. 2. *Immunoprecipitation of Uukuniemi virus-specific proteins from infected cells at various times after infection. Virus-infected chicken embryo cells were pulse-labeled for 1 h at various times after infection as shown in Fig. 1. Samples of the extracts were incubated with antiserum prepared against purified virions and the immunocomplexes collected by adsorption to fixed S. aureus. The precipitates were analyzed on a 10% polyacrylamide gel. (k) Immunoprecipitation of mock-infected cell extracts. (a through i) Immunoprecipitation of infected extracts labeled at 4, 6, 8, 10, 14, 16, 20, 22 and 26 h p.i., respectively. The positions of the virion marker proteins G1, G2, and N are as indicated.*

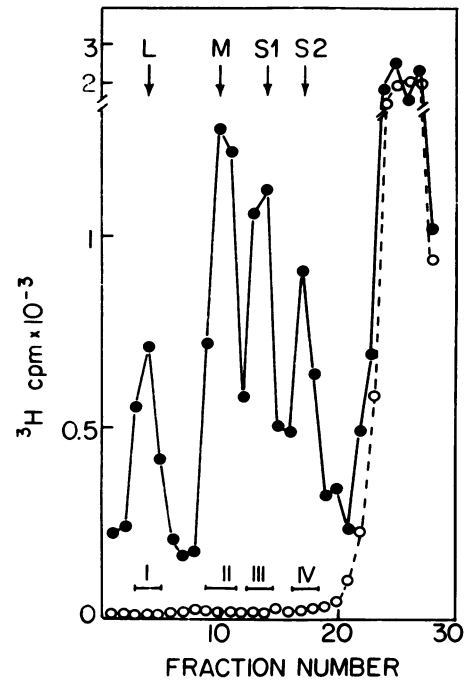

FIG. 3. *Fractionation of Uukuniemi virus-specific RNAs by sucrose gradient centrifugation. Uukuniemi virus-specific RNAs, labeled with [^{3}H]uridine in the presence of actinomycin D were isolated from infected chicken embryo cells at 8 h p.i. The cytoplasmic extract was analyzed on a 15 to 30% sucrose gradient (25,000 rpm, 17 h, 23°C, SW27 rotor). An extract from mock-infected cells was analyzed on a separate gradient. The positions of Uukuniemi virion RNA segments run in a separate gradient are indicated as L, M, and S1. Fractions (1 ml) were collected and trichloroacetic acid-precipitable radioactivity determined from samples of each fraction. Direction of sedimentation is from the right to the left. Peak fractions were pooled as indicated (I through IV) and used in subsequent translation assays. Symbols: ●, RNA from infected cells; ○, RNA from uninfected cells.*

fore prepared at 8 h p.i. Infected and mock-infected cells were labeled with [^{3}H]uridine from 4 to 8 h p.i. in the presence of actinomycin D (which does not inhibit Uukuniemi virus replication [20]). Cytoplasmic extracts were prepared, and the RNA was phenol extracted and analyzed by sucrose gradient centrifugation (Fig. 3). Four major peaks of acid-precipitable radioactivity were obtained in addition to some labeled material left on top of the gradient. The three fastest-sedimenting RNAs were sensitive to pancreatic RNase (Table 1) and cosedimented with the L (29S), M (23S), and S (17S) virion RNA species run in a separate gradient. The slowest-sedimenting RNA species (12S) was likewise RNase sensitive (Table 1) and absent from virions. The RNA species were designated L, M, S1, and S2, respectively. Mock-infected cells yielded no labeled radioactivity at the positions of the four RNA species, whereas the amount of labeled material left on top of the gradient was the same as in the infected cell extract.

To get an idea of the polarity of the cytoplasmic RNA species, their sensitivity to digestion with RNase was determined after self-annealing and annealing to an excess of total virion RNA (Table 1). For these experiments, the RNA was labeled from 1.5 to 8 h p.i. followed by fractionation of the RNA on sucrose gradients. The L and M RNAs contained both plus and minus strands as shown by self-annealing, whereas S1 and S2 self-annealed poorly. Annealing to an excess of virion RNA rendered about half of the L and M RNAs RNase resistant, whereas S2 was almost completely rendered RNase resistant. S1 did not efficiently hybridize to virion RNA. Unlabeled virion RNA mixed with trace amounts of ^{32}P-labeled virion RNA was fractionated on a sucrose gradient, and the individual RNA segments were recovered by ethanol precipitation. Hybridization of the virion RNA segments to the individual [^{3}H]uridine-labeled cytoplasmic RNA species showed that the cytoplasmic L RNA hybridized to the L segment (45.3% RNase resistance), the cytoplasmic M

TABLE 1. *Polarity of virus-specific cytoplasmic RNAs*

Conditions	Cytoplasmic RNA species	Acid-precipitable radioactivity (cpm)		RNase resistance (%)
		−RNase	+RNase	
Control (no melting, no annealing)	L	1,502	27	1.8
	M	1,979	46	2.3
	S1	1,157	5	0
	S2	1,489	3	0
Melted, unannealed	L	1,392	35	2.5
	M	1,931	20	1.5
	S1	977	3	0
	S2	1,401	2	0
Self-annealed	L	1,226	410	33.4
	M	1,776	601	33.8
	S1	1,114	158	14.2
	S2	956	83	8.7
Annealed to excess of virion RNA (5 μg)	L	1,280	682	53.3
	M	1,916	1,010	52.7
	S1	960	198	21.7
	S2	912	758	83.1

RNA hybridized to the M segment (48.1%), and the S2 RNA hybridized to the S segment (78.3%). The cytoplasmic S1 RNA did not hybridize to the virion S RNA (18.8% resistance).

In vitro translation of the RNA species. The peak fractions of each RNA species and corresponding fractions from mock-infected gradients were pooled individually and are herein referred to as pools I, II, III, and IV. After ethanol precipitation, the RNAs were used for in vitro translation assays in reticulocyte lysates (Fig. 4A through D).

Each of the four RNA pools directed the synthesis of a number of polypeptides, most of which were translated from RNA isolated from both infected and mock-infected extracts. Since the L protein (molecular weight, 180,000 to 200,000) is too large to be encoded by the M and S segments, it has to be a product of the L segment. Although polypeptides larger than 200,000 daltons were synthesized from RNAs in pool I (Fig. 4A), no polypeptide identifiable as the L protein was detected; there was no difference between the infected and mock-infected extracts (Fig. 4A, lanes a and b), and no polypeptides were immunoprecipitated with antivirion serum (Fig. 4A, lane c).

From infected pool II (Fig. 4B) a polypeptide with an apparent molecular weight of about 110,000 (relative to several molecular weight markers) was synthesized (Fig. 4B, lane b, arrow). This polypeptide was absent in the lysate programmed with the mock-infected pool II (Fig. 4B, lane a) and was selectively precipitated with antivirion serum (Fig. 4B, lane c, arrow). We designated this polypeptide p110.

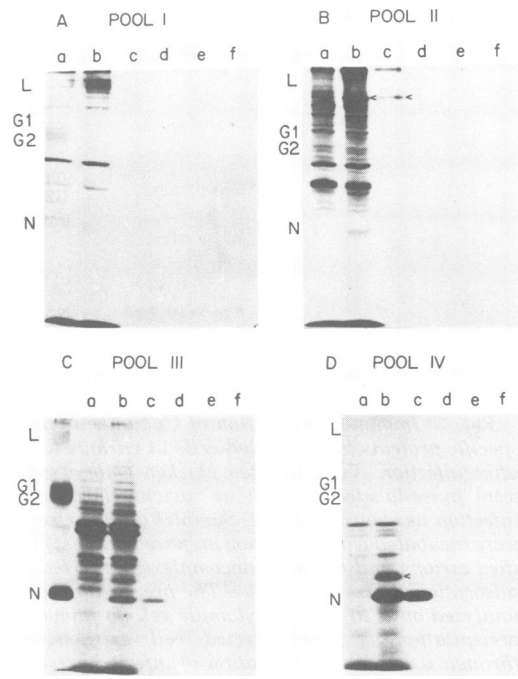

FIG. 4. *Cell-free translation of pool I through IV RNAs (Fig. 1) isolated from infected and mock-infected cells. RNAs fractionated on sucrose gradient were translated in cell-free micrococcal nuclease-treated reticulocyte lysates. Translation of RNA from mock-infected cells (a) and immunoprecipitation of the products with antiserum (e) and preimmune serum (f). Translation of RNA from infected cells (b) and precipitation with antiserum (c) and preimmune serum (d). The positions of the virion protein markers are indicated on the left of each panel. Arrows in (B) (lanes b and c) indicate the position of the 110,000-dalton polypeptide (p110) and in (D) (lane b) that of the NS 30,000-dalton polypeptide.*

The RNA from infected and mock-infected pools III (Fig. 4C) yielded similar polypeptide patterns. The only difference was a polypeptide made from the RNA of infected pool III that comigrated with the virion N protein (Fig. 4C, lane b). This product, which was precipitated with antivirion serum (Fig. 4C, lane c), is probably translated from a contaminating mRNA species present in pool IV (see below).

A major in vitro product that comigrated with the virion N protein was synthesized from RNA in the infected pool IV (Fig. 4D, lane b). It was readily precipitated with antivirion serum Fig. 4D, lane c) and was absent from the lysate programmed with RNA from the corresponding mock-infected pool IV (Fig. 4D, lane a). Roughly half of the incorporated radioactivity could be recovered in this product. In addition, a product migrating slightly slower than the N protein

(molecular weight, ~30,000) was synthesized from the infected pool IV (Fig. 4D, lane b, arrow). This product was absent from mock-infected pool IV (Fig. 4D, lane a), did not comigrate with any of the structural polypeptides, and was not precipitated with virion antiserum (Fig. 4D, lane c). We therefore called it NS (nonstructural).

Processing of p110 by dog pancreas microsomes in vitro. None of the RNA pools directed the synthesis of polypeptides of the size of G1 or G2. Instead, a 110,000-dalton polypeptide (p110) was made from the infected pool II RNA (M RNA fraction) (Fig. 4B, lanes b and c). In the case of snowshoe hare virus, a serologically unrelated bunyavirus, the M RNA has been shown to encode the two glycoproteins G1 (molecular weight, 115,000) and G2 (molecular weight, 38,000) (11). It was therefore conceivable that the Uukuniemi virus glycoproteins also are encoded by an mRNA with the size of M RNA and that the glycoproteins in fact are synthesized as a precursor, which posttranslationally is cleaved roughly in the middle. To test this possibility, we translated the pool II RNA in the presence and absence of dog pancreas microsomes to see whether p110 would be processed. Microsomal membranes, when added to in vitro translation lysates have been shown to facilitate the glycosylation and processing of membrane proteins of several enveoped RNA viruses during the translation event (8, 9, 26, 29). p110 was present in the initial translation product mixture (Fig. 5, lane c), and was selectively immunoprecipitated with antiserum (Fig. 5, lane d, arrow). In the presence of membranes, p110 quantitatively disappeared, and a new immunoprecipitable band appeared that migrated between the G1 and G2 virion markers (Fig. 5, lane e, arrow). Thus, addition of membranes resulted in the cleavage of p110 roughly in the middle.

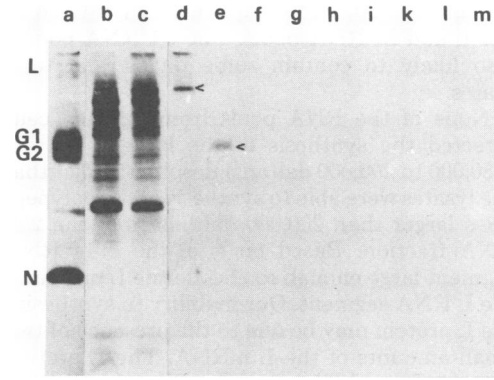

FIG. 5. *Cell-free translation of pool II RNA in the presence and absence of dog pancreas microsomal membranes. Translation of mock-infected (b) and infected (c) RNA in the absence of membranes. Products synthesized by RNA from infected cells in the absence (d) and presence (e) of membranes were immunoprecipitated with antivirion serum. Control immunoprecipitations were products precipitated with antiserum from lysates incubated with RNA from mock-infected cells in the absence (f) and presence (g) of membranes. (h through l) Same as in (d through g) but precipitated with preimmune serum; (m) incubation of reticulocyte lysate with membranes without added RNA; (a) [^{35}S]methionine-labeled Uukuniemi virion. Arrows indicate the position of the 110,000-dalton polypeptide (p110) (d), and its cleavage products (e).*

DISCUSSION

In this study, we have fractionated Uukuniemi virus-specific RNAs from infected cells and translated them in cell-free reticulocyte lysates. Two of the four RNA species found in sucrose gradients directed the synthesis of virus-specific polypeptides, namely, an mRNA species cosedimenting with the virion M RNA (23S), which gave rise to a novel 110,000-dalton protein (p110), and a small 12S mRNA species, called S2, which directed the synthesis of the virion N protein and a 30,000-dalton NS protein not found in virions.

Uukuniemi virion RNAs have a negative (anti-mRNA) polarity (25) and does not stimulate in vitro protein synthesis (unpublished data). Thus, virus-specific mRNA's have to be isolated from infected cells. Attempts to isolate polyadenylate-containing mRNA's from infected cells by oligodeoxythymidylic acid-cellulose chromatography have so far been unsuccessful (Pettersson et al., unpublished data). Therefore, the isolation of mRNA's by this method was not possible. Instead, we fractionated total cytoplasmic RNA from infected and mock-infected cells on sucrose gradients and translated different size fractions to identify virus-specific mRNA species and products synthesized by them. By this method, four virus-specific RNA species were identified, sedimenting at 29S (L), 23S (M), 17S (S1), and 12S (S2).

Annealing experiments suggested that the L and M RNA species consisted of RNA of both positive and negative polarities, whereas most of the S1 RNA appeared to be of the same polarity as the virion S segment. The S2 RNA efficiently hybridized to total virion RNA as well as the isolated S RNA segment, indicating that it is transcribed from this segment. The RNA in the cytoplasmic L and M pools partly annealed to the corresponding virion RNA segments. The lack of complete annealing in the various experiments is probably due to the complex mixture of full-sized virion RNA, full-sized complementary RNA (template for the replication of new

minus strands), and complementary mRNA in the L and M RNA pools. The S1 RNA pool is also likely to contain some plus-strand templates.

None of the RNA pools from infected cells directed the synthesis of the large L protein (180,000 to 200,000 daltons) despite the fact that the lysates were able to synthesize host polypeptides larger than 200,000 daltons from the 29S RNA fraction. Based on size, the only RNA segment large enough to encode the L protein is the L RNA segment. Our inability to synthesize the L protein may be due to the presence of two small amounts of the L mRNA. The L protein is not detectable in infected cells and is present in virions in very small quantities (16; Pettersson, unpublished data). Another possibility is that the mRNA and virion RNA may have formed hybrids during the isolation procedure thereby inactivating the mRNA as a template for translation.

An mRNA species cosedimenting with the virion M RNA stimulated the synthesis of a novel virus-specific 110,000-dalton polypeptide. When translation was carried out in the presence of dog pancreas microsomes, the p110 was absent, and a new polypeptide band migrating between the G1 and G2 glycoproteins appeared instead. Both polypeptides were immunoprecipitated with antivirion serum and were absent from lysates incubated with corresponding RNA from mock-infected cells. Thus, they must be virus specific. We interpret the results to mean that the glycoproteins are synthesized as a 110,000-dalton precursor in the absence of membranes, whereas in the presence of membranes this precursor is cleaved roughly in the middle. The fact that the cleaved products migrated as one band between G1 and G2 could be due to incomplete glycosylation (especially lack of sialic acid) with altered mobility as a consequence. In vitro posttranslational modifications by microsomal membranes have previously been shown to occur for four other enveloped RNA viruses. Vesicular stomatitis virus glycoprotein (G) is glycosylated in two discrete steps during translation of the G mRNA in vitro in the presence of microsomes (26). Under similar conditions, the Semliki Forest virus glycoprotein precursor, p97, is nascently cleaved to E1 (52,000 daltons) and p62 (the precursor of E3 and E2), which similarly are glycosylated and inserted into the endoplasmic membrane (9). The glycoproteins of Sindbis virus, which is closely related to Semliki Forest virus, are synthesized in the same way (29). In the case of influenza virus, the hemagglutinin (HA) is synthesized as an unglycosylated 63,000-dalton precursor in the absence of membranes and is converted to a glycosylated 75,000-dalton polypeptide in the presence of membranes (8). In contrast to what appears to be the case with Uukuniemi virus glycoproteins, the influenza HA precursor is not cleaved to the final products HA_1 and HA_2 during the translation process. Instead, the cleavage takes place at the plasma membrane during the final maturation stage (12). Our results regarding the role of microsomal membranes in the modification of Uukuniemi virus glycoproteins have to be considered preliminary. More detailed analyses with methods described for the above viruses have to be done to elucidate these complex events.

The coding capacity of the M RNA has been estimated to be about 110,000 to 120,000 daltons of protein (21). The size of the precursor (p110) therefore suggests that the M segment is large enough to encode the two glycoproteins and that the genetic capacity of this segment is efficiently used. Our results agree with previous observations by Gentsch and Bishop (11) who showed by genetic recombination between temperature-sensitive mutants of two bunyaviruses that the M RNA segment of snowshoe hare virus encodes both glycoproteins.

Uukuniemi virus N protein was translated from an mRNA species smaller than any of the virion RNA segments (12S). The same RNA fraction also directed the synthesis of a 30,000-dalton polypeptide, which was not found in virions, was not precipitated by virion antiserum, and was absent from lysates programmed with the corresponding size-fraction RNA from mock-infected extracts. In addition, a polypeptide with similar mobility was seen in extracts from pulse-labeled infected cells from about 8 h p.i. This protein, which we call NS, may therefore represent a nonstructural polypeptide. The preliminary hybridization experiments suggest that the S2 RNA(s) is transcribed from the virion S RNA. The fact that in the case of snowshoe hare virus the N protein is translated in vitro from an mRNA derived from the S RNA (10) supports this conclusion. No evidence for a similar NS protein was, however, found with snowshoe hare virus. The Uukuniemi virus S RNA segment has been estimated to have a molecular weight of about $0.5 \pm 0.1 \times 10^6$ (21) and therefore the genetic capacity to encode a protein of roughly 50,000 daltons. The estimated combined size of N and NS is about 55,000 daltons. Thus, the coding capacity of the S RNA would be fully used. We do not know yet whether the N and the NS proteins are translated from two different mRNA's of roughly the same size, or from the same 12S mRNA. The situation with the S RNA segment of Uukuniemi virus resembles that of the eighth segment of influenza virus. It has recently been shown (15) that the smallest

(eighth) RNA segment of influenza virus is transcribed into two separate mRNA's, each coding for one of the NS polypeptides NS_1 and NS_2. The mRNA for the NS_2 (about 340 nucleotides) is identical to the 3' portion of the NS_1 mRNA (about 860 nucleotides), and the proteins are translated from the mRNA's partly in different reading frames (15). A similar situation could also exist in the case of Uukuniemi virus S RNA segment. Another possibility would be that the Uukuniemi virus NS protein is a precursor of the N protein. This is highly unlikely, since the NS is not precipitated with antivirion serum, which efficiently precipitates the N protein. Lack of sufficient material has so far prevented comparison of the two proteins by tryptic peptide analysis or partial proteolysis.

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LITERATURE CITED

1. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell. Biol. **67**:835–851.
2. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. II. Reconstruction of functional rough microsomes from heterologous components. J. Cell. Biol. **67**:852–862.
3. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
4. **Bouloy, M., and C. Hannoun.** 1976. Studies on Lumbo virus replication. I. RNA-dependent RNA polymerase associated with virions. Virology **69**:258–264.
5. **Bouloy, M., S. Krams-Ozden, F. Horodniceanu, and C. Hannoun.** 1973–1974. Three-segment RNA genome of Lumbo virus. Intervirology **2**:173–180.
6. **Cash, P., A. C. Vezza, J. R. Gentsch, and D. H. L. Bishop.** 1979. Genome complexities of the three mRNA species of snowshoe hare bunyavirus and in vitro translation of S mRNA to viral N polypeptide. J. Virol. **31**: 685–694.
7. **Clewley, J., J. Gentsch, and D. H. L. Bishop.** 1977. Three unique viral RNA species of snowshoe hare and La Crosse bunyaviruses. J. Virol. **22**:459–468.
8. **Elder, K. T., J. M. Bye, J. J. Skehel, M. D. Waterfield, and A. E. Smith.** 1979. In vitro synthesis, glycosylation, and membrane insertion of influenza virus haemagglutinin. Virology **95**:343–350.
9. **Garoff, H., K. Simons, and B. Dobberstein.** 1978. Assembly of Semliki Forest virus membrane glycoproteins in the membrane of the endoplasmic reticulum in vitro. J. Mol. Biol. **124**:587–600.
10. **Gentsch, J. R., and D. H. L. Bishop.** 1978. Small viral RNA segment of bunyaviruses codes for viral nucleocapsid protein. J. Virol. **28**:417–419.
11. **Gentsch, J. R., and D. H. L. Bishop.** 1979. M viral RNA segment of bunyaviruses codes for two glycoproteins, G1 and G2. J. Virol. **30**:767–770.
12. **Hay, A. J.** 1974. Studies on the formation of the influenza virus envelope. Virology **60**:398–418.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Lamb, R. A., and P. W. Choppin.** 1979. Segment 8 of the influenza virus genome is unique in coding for two polypeptides. Proc. Natl. Acad. Sci. U.S.A. **76**:4908–4912.
15. **Lamb, R. A., P. W. Choppin, R. M. Chanock, and C.-H. Lai.** 1980. Mapping of the two overlapping genes for polypeptides NS_1 and NS_2 on RNA segment 8 of influenza virus genome. Proc. Natl. Acad. Sci. U.S.A. **77**: 1857–1861.
16. **Obijeski, J. F., D. H. L. Bishop, F. A. Murphy, and E. L. Palmer.** 1976. Structural proteins of La Crosse virus. J. Virol. **19**:985–997.
17. **Obijeski, J. F., and F. A. Murphy.** 1977. Bunyaviridae: recent biochemical developments. J. Gen. Virol. **37**:1–14.
18. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
19. **Persson, H., U. Pettersson, and M. B. Mathews.** 1978. Synthesis of a structural adenovirus polypeptide in the absence of viral DNA replication. Virology **90**:67–79.
20. **Pettersson, R. F.** 1974. Effect of Uukuniemi virus infection on host cell macromolecule synthesis. Med. Biol. **52**:90–97.
21. **Pettersson, R. F., M. J. Hewlett, D. Baltimore, and J. M. Coffin.** 1977. The genome of Uukuniemi virus consists of three unique RNA segments. Cell **11**:51–63.
22. **Pettersson, R., and L. Kääriäinen.** 1973. The ribonucleic acids of Uukuniemi virus, a non-cubical tick-borne arbovirus. Virology **56**:608–619.
23. **Pettersson, R., L. Kääriäinen, C.-H. von Bonsdorff, and N. Oker-Blom.** 1971. Structural components of Uukuniemi virus, a non-cubical tick-borne arbovirus. Virology **46**:721–729.
24. **Porterfield, J. S., J. Casals, M. P. Chumakov, S. Y. Gaidamovich, C. Hannoun, I. Holmes, M. C. Horzinek, M. Mussgay, N. Oker-Blom, and P. K. Russell.** 1975–1976. Bunyaviruses and bunyaviridae. Intervirology **6**:13–14.
25. **Ranki, M., and R. F. Pettersson.** 1975. Uukuniemi virus contains an RNA polymerase. J. Virol. **16**:1420–1425.
26. **Rothman, J. E., and H. F. Lodish.** 1977. Synchronized trans-membrane insertion and glycosylation of a nascent membrane protein. Nature (London) **269**:775–780.
27. **Villa-Kamaroff, L., M. McDowell, D. Baltimore, and H. F. Lodish.** 1974. Translation of reovirus mRNA, poliovirus RNA and bacteriophage QβRNA in cell-free extracts of mammalian cells. Methods Enzymol. **30**: 709–723.
28. **von Bonsdorff, C.-H., and R. Pettersson.** 1975. Surface structure of Uukuniemi virus. J. Virol. **16**:1296–1307.
29. **Wirth, D. F., F. Katz, B. Small, and H. F. Lodish.** 1977. How a single Sindbis virus mRNA directs the synthesis of one soluble protein and two integral membrane glycoproteins. Cell **10**:253–263.