JOURNAL OF VIROLOGY, Feb. 1984, p. 403–408
0022-538X/84/020403-06$02.00/0

Vol. 49, No. 2

Rubella Virus 40S Genome RNA Specifies a 24S Subgenomic mRNA That Codes for a Precursor to Structural Proteins

CHRISTIAN OKER-BLOM, ISMO ULMANEN, LEEVI KÄÄRIÄINEN, AND RALF F. PETTERSSON*

Recombinant DNA Laboratory, University of Helsinki, SF-00380 Helsinki 38, and Department of Virology, University of Helsinki, SF-00290 Helsinki 29, Finland

Received 19 August 1983/Accepted 24 October 1983

We have analyzed the structure of the rubella virus genome RNA and the virus-specific RNA species synthesized in B-Vero cells infected with rubella virus. A single-stranded, capped, and polyadenylated RNA species sedimenting at 40S in a sucrose gradient was released from purified virions treated with sodium dodecyl sulfate. This RNA species migrated with an M_r of about 3.8×10^6 in an agarose gel after denaturation with glyoxal and dimethyl sulfoxide. Infected cells labeled with [^{3}H]uridine in the presence of actinomycin D contained, in addition to the 40S RNA, a single-stranded polyadenylated 24S RNA species as shown by sucrose gradient analysis. In a Northern blot analysis, this RNA hybridized to a cDNA probe derived from the 3′ portion of the genomic 40S RNA. In vitro translation of the 24S RNA species yielded a 110,000-dalton polypeptide, in addition to some smaller products which were immunoprecipitated with an antiserum prepared against the structural proteins E1, E2a, E2b, and C. Since the sum of the molecular weights of the nonglycosylated envelope proteins and the capsid protein has been estimated to be about 116,000 (C. Oker-Blom et al., J. Virol. **46**:964–973, 1983), these results suggest that the 24S RNA species represents a subgenomic mRNA coding for a precursor (p110) to the structural proteins of rubella virus. Thus, the strategy of gene expression of rubella virus appears to be similar to that of the alphaviruses.

The molecular biology of rubella virus (RV), the sole member of the genus *Rubivirus* of the *Togaviridae* family (18), has been rather poorly characterized (4, 5). This is especially true for the synthesis of virus-specific RNAs, the details of which are largely unknown. We have recently found that purified RV contains one capsid protein, C (M_r = 33,000), and three envelope glycoproteins, E1 (M_r = 58,000), E2a (M_r = 47,000), and E2b (M_r = 42,000) (14). Similar results have recently been reported also by Waxham and Wolinsky (25). Our tryptic peptide analyses indicated that E2a and E2b are very closely related and may represent two glycosylation variants of the same gene product (14). The molecular weights of the unglycosylated E1 and E2 synthesized in RV-infected cells in the presence of tunicamycin were found to be about 53,000 and 30,000, respectively. Thus, the structural genes must have a coding capacity for at least 116,000 daltons of protein.

Previous results have indicated that the genome of RV consists of a 40S single-stranded RNA (6–8, 19, 22), which is infectious (7) and thus must have a positive polarity (1). This RNA species was also found in RV-infected BHK cells (6, 8, 19, 22). Whether a subgenomic mRNA equivalent to the 26S mRNA found in alphavirus-infected cells (9) is also synthesized in RV infected cells has remained an open question (4–6).

In this paper, we have characterized the virus-specific RNAs found in purified RV and in RV-infected B-Vero cells. Evidence is presented that infected cells contain, in addition to the genomic 40S RNA, a subgenomic 24S RNA that is derived from the 3′ end of the 40S RNA and encodes a 110,000-dalton polypeptide, which probably represents the precursor to the structural proteins.

MATERIALS AND METHODS

Virus and cells. The Therien strain of RV, originally obtained from Ann Schluedeberg, Yale University, New Haven, Conn., was grown in B-Vero cells in 2-liter glass roller bottles in the presence of Eagle minimum essential medium supplemented with 0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and with 100 IU of penicillin per ml, 50 μg of streptomycin per ml, and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.2). High titer rubella stock virus was prepared after three consecutive plaque purification steps from a single plaque by low multiplicity of infection (14). The final stock virus had a titer of 2×10^8 PFU/ml and a hemagglutination titer of 128. Virus was cultivated, concentrated, and purified as described previously (14).

Radioactive virus and purification of virion RNA. To obtain ^{32}P-labeled virus, RV was cultivated in B-Vero cells grown in roller bottles in the presence of ^{32}P (0.5 mCi/ml; The Radiochemical Centre, Amersham, England), harvested, and purified as described previously (14).

A 2-mg amount of purified unlabeled RV and 5×10^5 cpm of ^{32}P-labeled RV were mixed, solubilized with 2% sodium dodecyl sulfate (SDS), and layered on a 15 to 30% sucrose gradient in TNE buffer (0.1 M NaCl, 50 mM Tris [pH 7.4], 1 mM EDTA) containing 0.1% SDS. Centrifugation was for 12 h at 23,000 rpm at 23°C in an SW27 rotor. ^{32}P-labeled Semliki Forest virus (SFV) 42S and 26S RNAs and 28S and 18S rRNAs were centrifuged in separate tubes as markers. To localize the RNA, 0.5-ml fractions were collected from below and assayed for radioactivity by Cherenkov counting. Peak fractions were pooled, and the RNA was concentrated by ethanol precipitation.

Isolation of cytoplasmic RNA. B-Vero cells grown in roller bottles were infected with 5 PFU per cell. At 20 and 21 h postinfection, actinomycin D (2 μg/ml) and [^{3}H]uridine (50 μCi/ml) (The Radiochemical Centre), respectively, were added. At 25 h, cells were washed twice with phosphate-buffered saline and trypsinized (0.5% trypsin and 0.1% EDTA) for 5 min at 37°C. Cells were then washed with ice-cold RSB-Na$^+$ (0.01 M Tris [pH 7.4], 0.02 M NaCl, 0.0015 M $MgCl_2$) in the presence of 40 μg of polyvinylsulfate per ml. The cells were allowed to swell for 10 min and were

* Corresponding author.

disrupted by 20 strokes in a tight-fitting Dounce homogenizer. The nuclei were pelleted at 1,000 rpm for 5 min, and SDS and sodium acetate were added to the supernatant to final concentrations of 2% and 0.3 M, respectively. The RNA was then extracted twice with a mixture of buffer-saturated phenol-chloroform-isoamyl alcohol (25:24:1). Part of this RNA was directly layered on a sucrose gradient (see below). From the rest, polyadenylated [poly(A)$^+$] RNA was prepared by oligodeoxythymidylic acid [oligo(dT)] cellulose (P-L Biochemicals, Inc., Milwaukee, Wis.) chromatography by standard procedures. Total and poly(A)$^+$ RNA were fractionated on a 15 to 30% sucrose gradient in an SW27 rotor at 23,000 rpm for 14 h at 23°C. The RNA-containing peak fractions were pooled separately, and the RNA precipitated with ethanol in the presence of yeast tRNA (20 μg/ml; Sigma).

To determine the sensitivity of the RNA to RNase, samples from each fraction of a gradient lacking SDS were treated with RNase A (Worthington Diagnostics, Freehold, N.J.) (50 μg/ml) for 30 min at 37°C in TNE buffer. Equal samples were incubated without RNase. Trichloroacetic acid-precipitable radioactivity was then determined for each fraction.

Agarose gel electrophoresis. Samples of ^{32}P-labeled virion 40S RNA, SFV 42S and 26S, or 28S and 18S rRNAs (used as markers) were denatured in 1 M glyoxal and 50% dimethyl sulfoxide for 60 min at 50°C by the method of McMaster and Carmichael (13). The RNAs were then fractionated by electrophoresis in a 1% agarose gel with a 0.01 M NaH_2PO_4 (pH 7.0) running buffer.

Northern blot analysis. Samples of [^{3}H]uridine-labeled RNA were denatured and fractionated on 1% agarose gels as described above. The RNA was transferred to nitrocellulose paper and prehybridized as described by Thomas (24). The filters were incubated with a ^{32}P-labeled cDNA probe prepared from RV 40S RNA as described previously (23). The reaction mixture (100 μl) contained 50 mM Tris-hydrochloride (pH 8.3), 60 mM KCl, 12 mM $MgCl_2$, 1 mM dithiothreitol, 0.8 mM each of dATP, dTTP, and dGTP, 0.1 mM of dCTP, 160 μCi of [α-^{32}P]dCTP (400 Ci/mmol; Amersham), 10 μg (about 2.5 pmol) of RV RNA as template, and 5 μg of oligo(dT)$_{12\text{-}18}$ (P-L Biochemicals) as primer. Incubation was for 30 min at 42°C with 400 U/ml of reverse transcriptase from avian myeloblastosis virus (J. Beard, Life Sciences, Inc., St. Petersburg, Fla.). The cDNA was purified on a Bio-Gel P30 column and concentrated by ethanol precipitation. The cDNA had a specific activity of about 3×10^7 cpm/μg and a mean length of about 900 nucleotides and maximum length of about 3,000 nucleotides as analyzed by electrophoresis on an alkaline agarose gel (12) (data not shown). Filter hybridization was carried out as described by Thomas (24) in 50% formamide at 42°C overnight with 0.5×10^6 cpm/ml of the probe. The filters were washed extensively, dried, and autoradiographed at −70°C with an intensifying screen.

The 5′ end cap analysis. The 5′ end structure of RV RNA was analyzed as described in detail previously (16, 17, 20, 21). Shortly, ^{32}P-labeled RV 40S RNA (4×10^6 cpm) or SFV 42S RNA (3×10^6 cpm) (used as a control) were completely digested for 2 h at 37°C with a mixture of RNases T1 (200 U/ml; Calbiochem, La Jolla, Calif.), T2 (50 U/ml; Calbiochem), and A (150 μg/ml; Worthington) in 10 mM Tris (pH 7.5)–1 mM EDTA. The products were separated by ionophoresis on DEAE-cellulose paper at pH 3.5 (2). The radiolabeled compounds were located by autoradiography. The tentative 5′-end structures were eluted from the paper by 30% triethylamine bicarbonate (pH 8.0), lyophilized, and further digested with bacterial alkaline phosphatase (BAP) (Worthington) for 1 h at 37°C in 5 μl of 10 mM Tris (pH 7.5) containing 0.01 U of enzyme and 5 μg of carrier tRNA (16, 17, 20). The products were analyzed by ionophoresis on Whatman 3MM paper at pH 3.5, followed by autoradiography.

In vitro translation. RNA pooled from sucrose gradient fractions and concentrated by ethanol precipitation was resuspended in water and translated in a micrococcal nuclease-treated rabbit reticulocyte lysate by the method of Pelham and Jackson (15) with commercial reagents (Amersham). Translation was done in a 75-μl reaction volume with 0.5 μCi of [^{35}S]methionine per μl (1.455 Ci/mmol) and incubated at 30°C for 60 min. The samples were then immunoprecipitated as described below and previously (14).

Immunoprecipitation. Samples from in vitro translation mixtures were diluted into 0.5 ml of NET buffer (1% Nonidet P-40, 0.4 M NaCl, 0.05 M Tris [pH 8.0], 0.005 M EDTA) containing 100 IU of Trasylol (Bayer, Leverkusen, West Germany). A 5-μl amount of antiserum against RV structural proteins or control preimmune serum was added, and the mixtures were incubated at 4°C overnight. Protein A Sepharose (CL-4B; Pharmacia Fine Chemicals, Uppsala, Sweden) was added, and the samples were incubated for 4 h at 4°C. The precipitates were concentrated by centrifugation and washed three times with NET buffer and once with water. Samples were then boiled in electrophoresis sample buffer (10), and supernatants were analyzed by 5 to 16% gradient SDS-gel electrophoresis (10, 14) followed by autoradiography (3). The ^{14}C-methylated molecular weight markers used were myosin (M_r = 200,000), BSA (M_r = 69,000), ovalbumin (M_r = 46,000), carbonic anhydrase (M_r = 30,000), and lysozyme (M_r = 14,300) (all from Amersham). Purified [^{35}S]methionine-labeled RV proteins were also used as markers.

RESULTS

Characterization of virion RNA. Purified ^{32}P-labeled RV was disrupted with 2% SDS and analyzed by sucrose gradient centrifugation (Fig. 1). A prominent peak sedimenting at about 40S (11) relative to the marker RNAs used was obtained. This RNA species probably represents the genomic RNA (6–8, 19, 22). A minor peak cosedimenting with the SFV 26S RNA marker was consistently observed. Occasionally, this RNA species was seen as a shoulder on the 40S RNA peak. The identity of this RNA species was not studied further. The labeled material remaining at the top of the gradient represents the phospholipids of the envelope. To determine the size of the 40S RNA more accurately, samples from the peak fraction (Fig. 1) as well as of the marker RNAs were treated with glyoxal and dimethyl sulfoxide (13) and analyzed by agarose gel electrophoresis (Fig. 2). The RV RNA (Fig. 2, lane 1) migrated slightly faster than the SFV 42S RNA (Fig. 2, lane 2), with an apparent molecular weight of about 3.8×10^6, relative to the four marker RNAs (Fig. 2, lanes 2 to 5). About 85% of the radioactivity of the 40S RNA peak fraction was bound to oligo(dT) cellulose, under conditions where less than 1% of the rRNAs and about 60% of SFV 42S RNA were bound, suggesting that RV 40S RNA contains a poly(A) tract.

To study whether the 40S RNA has a blocked 5′-end cap structure typical for eucaryotic mRNAs, uniformly ^{32}P-labeled 40S RNA or SFV 42S RNA was completely digested with a mixture of RNases T1, T2, and A. This treatment will degrade RNA to 3′ mononucleotides, but does not digest 5′-phosphorylated (e.g., pppNp) or capped and methylated

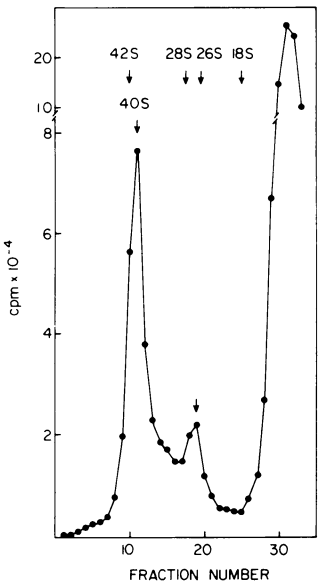

FIG. 1. Fractionation of RV virion RNA by sucrose gradient centrifugation. ^{32}P-labeled RV was treated with 2% SDS and layered on a 15 to 30% sucrose gradient. Centrifugation was for 12 h at 23,000 rpm in an SW27 rotor. ^{32}P-labeled SFV 42S and 26S RNAs and 28S and 18S RNAs were run as markers in separate tubes. Fractions of 0.5 ml were collected from the bottom and assayed for radioactivity. Arrow indicates the position of the 26S RNA species (see text).

termini because PP_i linkages and nucleotides with 2′-*O*-methylation are resistant (20). Figure 3A shows an autoradiogram from the analysis of the digestion products by ionophoresis at pH 3.5 on DEAE paper. The SFV 42S RNA (Fig. 3, lane 1) yielded, in addition to the monophosphates, the slowly migrating 5′ structure, which we have previously determined to be ^{7}mGpppAp (17). RV 40S RNA yielded two distinct slowly migrating products (Fig. 3, spots a and b), which had mobilities different from that of the SFV cap structure. In addition, some minor more rapidly migrating products and material left at the origin were observed. They probably represent incomplete digestion products, since

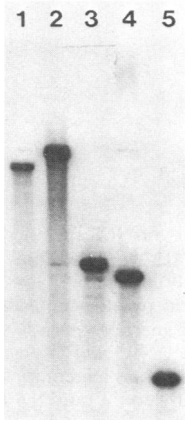

FIG. 2. Agarose gel electrophoresis of ^{32}P-labeled purified glyoxylated RV virion RNA (lane 1), SFV 42S virion RNA (M_r = 4.3 × 10^6; reference 9) (lane 2), 28S rRNA (M_r = 1.6 × 10^6) (lane 3), SFV 26S RNA (M_r = 1.4 × 10^6; reference 9) (lane 4), and 18S rRNA (M_r = 0.6 × 10^6) (lane 5).

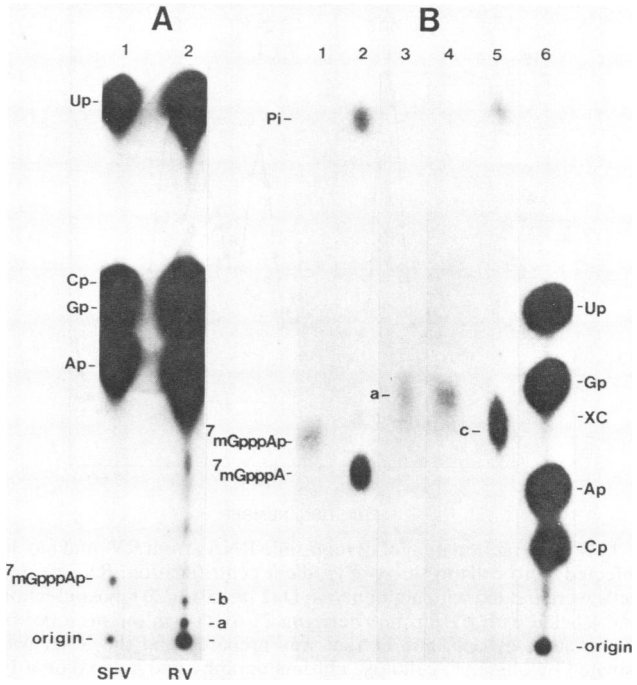

FIG. 3. Isolation and characterization of the 5′ terminus of RV 40S RNA. (A) Uniformly ^{32}P-labeled SFV 42S RNA (3 × 10^6 cpm, by Cherenkov counting) (lane 1) and RV 40S RNA (4 × 10^6 cpm) (lane 2) were completely digested with a mixture of RNases T1, T2, and A, and the products were separated by ionophoresis on DEAE paper at pH 3.5 (4 h, 20 V/cm). The paper was dried and autoradiographed. The 5′ terminus of SFV 42S RNA (^{7}mGpppAp; reference 17) and the tentative 5′ termini of RV 40S RNA (spots a and b) were eluted and treated with BAP. (B) The products were analyzed by ionophoresis on 3MM paper at pH 3.5 (90 min, 20 V/cm). Untreated (lane 1) and BAP-treated (lane 2) 5′ terminus of SFV 42S RNA; untreated (lane 3), RNase T1, T2, and A retreated (lane 4), and BAP-treated (lane 5, spot a). Cp, Ap, Gp, and Up indicate the position of the four 3′ monophosphates run as markers (lane 6). XC, position of xylene cyanol.

they yielded only monophosphates upon redigestion. The tentative 5′-end structures of RV RNA (Fig. 3, spots a and b) were eluted and further treated with BAP. The phosphatase should remove any external phosphate. Thus, all phosphate residues of a pppNp structure should be released as P_i, whereas only the 3′-terminal phosphate residue of a cap structure (e.g., ^{7}mGpppNp) should be released. As expected, BAP treatment of the SFV cap structure released P_i, leaving ^{7}mGpppA undigested (Fig. 3B, lane 2). P_i was also released from the structure in spot a by the treatment (Fig. 3B, lane 5) and resulted in a slight decrease in the mobility of spot a (Fig. 3B, lane 4) or spot a redigested with RNases T1, T2, and A (Fig. 3B, lane 3). Spot b of the RV RNA also released some P_i, whereas the rest of the material migrated as a broad smear slightly slower than the undigested material (data not shown). Thus, spots a and b both appeared to represent two different forms of a cap structure at the 5′ end of RV RNA.

Identification of a subgenomic RNA species. RV- or mock-infected cells were labeled with [^{3}H]uridine in the presence of actinomycin D at 21 to 25 h after infection, a time period corresponding to the late exponential phase of the release of infectious virus (unpublished data). Cells were disrupted, and cytoplasmic RNA was subjected to oligo(dT) cellulose

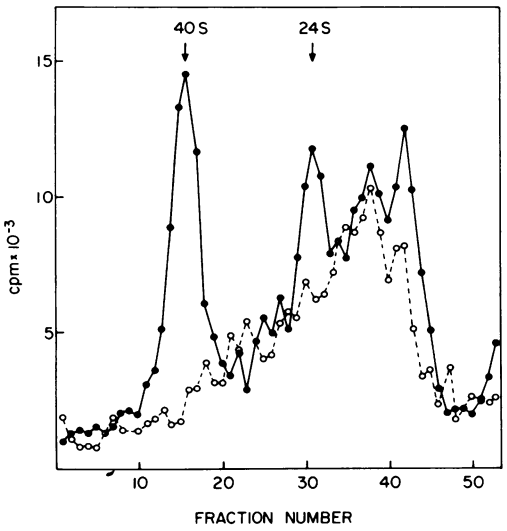

FIG. 4. Fractionation of cytoplasmic RNAs from RV- and mock-infected Vero cells by sucrose gradient centrifugation. RV-infected cells were treated with actinomycin D (2 μg/ml) at 20 h postinfection and labeled with [^{3}H]uridine between 21 to 25 h in the presence of the drug. A cytoplasmic extract was prepared and the RNA was isolated by oligo(dT) cellulose chromatography and layered on a 15 to 30% sucrose gradient containing 0.1% SDS. RNA from mock-infected cells was labeled and extracted similarly and analyzed on a separate gradient. ^{32}P-labeled RV 40S RNA was also run in a separate tube as marker. Centrifugation was for 14 h at 23,000 rpm at 23°C in an SW27 rotor. Fractions of 0.3 ml were collected from below and assayed for radioactivity. Symbols: ●, RNA from RV-infected cells; ○, RNA from mock-infected cells.

chromatography. The poly(A)$^+$ RNA was analyzed by sucrose gradient centrifugation (Fig. 4). A prominent RNA species cosedimenting with the 40S virion RNA was observed. In addition, a 24S RNA species was obtained. These RNA species were not present in extracts derived from mock-infected cells. A high background of labeled heterogeneous RNA was consistently obtained from both mock- and RV-infected cells, and the sedimentation profiles were reproducibly about the same (Fig. 4). Higher concentrations of actinomycin D (up to 5 μg/ml) and longer preincubations with the drug (up to 3 h) did not reduce the level of background radioactivity in the gradient. Except for a higher level of background radioactivity, essentially the same results were obtained with total cytoplasmic RNA, which had not been poly(A)-selected (data not shown). Almost all radioactivity in the fractions of gradients lacking SDS was rendered acid soluble by treatment with RNase A (data not shown), indicating that the RNA bound to oligo(dT) cellulose and recovered in the gradient was single stranded.

Total cytoplasmic RNA from RV- and mock-infected cells and from RNA bound to oligo(dT) cellulose was denatured, fractionated on an agarose gel, and transferred to nitrocellulose paper. The filter was then incubated with a ^{32}P-labeled cDNA probe made from the virion 40S RNA by reverse transcriptase with an oligo(dT) primer. The total cytoplasmic RNA contained two RNA species which hybridized with the probe; a large one, which comigrated with the virion 40S RNA marker, and an RNA species with an M_r of 1.2 × 10^6 (Fig. 5, lanes 4 and 6). No RNA species from the mock-infected cells hybridized with the probe (Fig. 5, lanes 5 and 7). Hybridization of the probe with RNAs taken from the 40S and 24S peak fractions of the sucrose gradient in Fig. 4 detected only the genomic 40S RNA (Fig. 5, lane 8) and the 1.2 × 10^6-dalton RNA (Fig. 5, lane 9). RNA from the corresponding fractions derived from the mock-infected cells did not hybridize with the probe (Fig. 5, lanes 10 and 11).

In vitro translation of the subgenomic 24S RNA. The subgenomic 26S mRNA of the alphaviruses encodes a 130,000-dalton precursor of the four structural proteins. To study whether the 24S RNA species similarly encodes the structural proteins of RV, the 24S RNA isolated from the sucrose gradient (Fig. 4) was translated in vitro in a micrococcal nuclease-treated rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The products were precipitated with antiserum raised against the structural proteins of RV (14). As shown in Fig. 6, a major product with an M_r of about 110,000 (p110) was observed by SDS-gel electrophoresis (indicated by arrow in Fig. 6, lane 1). In addition, four discrete products (M_r of about 88,000, 66,000, 62,000, and 20,000) and some minor immunoreactive products were identified. No products were precipitated from a lysate programmed with a 24S RNA fraction from a mock-infected extract (Fig. 6, lane 2). Preimmune serum did not precipitate any translation products (Fig. 6, lanes 3 and 4). Thus, we conclude that p110 contains antigenic determinants of the RV structural proteins.

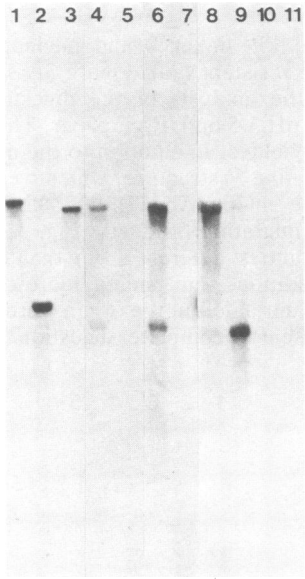

FIG. 5. Identification of RV-specific cytoplasmic RNAs by Northern blot analysis. RNAs to be analyzed were denatured with glyoxal and dimethyl sulfoxide, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose paper (13). ^{32}P-labeled SFV 42S and 26S RNAs and RV virion RNA (lanes 1 to 3) were run as markers. RV-specific RNAs were detected by hybridization to a ^{32}P-labeled cDNA probe complementary to the 3′ end of RV 40S RNA. Samples analyzed were: total RNA from RV-infected (lane 4) and mock-infected (lane 5) cells; total poly(A)$^+$ RNA from RV-infected (lane 6) and mock-infected (lane 7) cells; poly(A)$^+$ RNA from the 40S RNA peak (lane 8) and 24S RNA peak (lane 9) in Fig. 4, and the corresponding fractions of the gradient loaded with RNA from mock-infected cells (lanes 10 and 11) in Fig. 4. All samples were analyzed on the same gel, but the different lanes were exposed at different times.

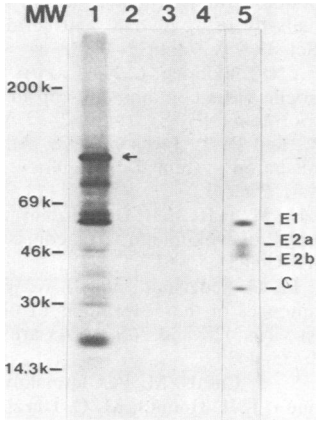

FIG. 6. SDS-gel analysis of immunoprecipitated products derived from in vitro-translated 24S RNA. A rabbit micrococcal nuclease-treated rabbit reticulocyte extract was programmed in the presence of [^{32}S]methionine with the subgenomic RV 24S RNA (see Fig. 4) or the corresponding fraction of mock-infected cells and analyzed by SDS-gel electrophoresis after immunoprecipitation with antiserum against RV-structural proteins (lanes 1 and 2, respectively) or preimmune serum (lanes 3 and 4). Lane 5, [^{35}S]methionine-labeled RV proteins. The positions of the molecular weight markers used (see text) are indicated on the left. Arrow indicates the position of the p110 translation product.

DISCUSSION

Here we have characterized the genomic RNA of RV and the cytoplasmic RNA species found in RV-infected B-Vero cells. The genome was found to consist of a single-stranded, polyadenylated and capped 40S RNA with an M_r of about 3.8×10^6. In addition to the 40S RNA, a 24S RNA species (M_r of about 1.2×10^6) of positive polarity was detected in infected cells. This RNA encoded a 110,000-dalton (p110) polypeptide, which probably represents the precursor of the structural proteins. Although we have not determined the exact structure of the 5′ terminus of RV 40S RNA nor the length and location of the poly(A) tract, our results indicate that the 40S RNA has a structure typical for eucaryotic mRNAs. The presence of the poly(A) tract was evident from the efficient binding of the RNA to oligo(dT) cellulose and the fact that cDNA was readily made with an oligo(dT) primer. We assume that the poly(A) tract is located at the 3′ end of the RNA. Two tentative 5′ termini were isolated after complete hydrolysis of the RNA. Both were resistant to the treatment with alkaline phosphatase as is typical for capped 5′-terminal structures (17, 20, 21). A pppNp terminus, typical for negative-strand viral RNAs, would have been completely sensitive to the treatment with alkaline phosphatase (16). Whether the two tentative cap structures of RV RNA represent two differently methylated forms similar to the ones found at the 5′ end of, e.g., vesicular stomatitis virus mRNAs (20), remains to be determined. Lack of sufficient amount of radioactivity did not allow us to study the detailed structure of the caps. A 38-41S RNA species of RV has previously also been demonstrated by others (6–8, 19, 22). Since this RNA was found to be infectious (7), it has been assumed that the RNA has a positive polarity (1). Our results presented here support this conclusion. We also detected regularly an RNA species sedimenting at 26S from purified RNA. Whether this RNA species represents a host cell contaminant, a specific degradation product of the genomic RNA, or a defective interfering RNA was not studied. The RNA did not cosediment with any of the rRNAs nor with the subgenomic 24S RNA, and it was not detected in infected cells.

A single-stranded subgenomic RV-specific 24S RNA species was detected in infected cells against a rather high background of ^{3}H-labeled heterogeneous, single-stranded, poly(A)$^+$ host cell RNA. We have not overcome the problem of the high background radioactivity, although we have tried several different procedures for the isolation of the RNA (unpublished data). Higher doses of actinomycin D or longer preincubations with the drug did not decrease the background. Apparently there are some poly(A)$^+$ RNA species in B-Vero cells whose synthesis is resistant to actinomycin D. Alternatively, actinomycin D may not penetrate the cells efficiently. That the 24S RNA is indeed a virus-specific subgenomic mRNA is based on the following evidence. (i) The RNA was not detected in mock-infected cells, (ii) it hybridized to a cDNA probe reverse transcribed from the genomic RNA, and (iii) it directed the in vitro synthesis of a 110,000-dalton (p110) polypeptide and some smaller products that were immunoprecipitated with a highly specific antiserum against RV structural proteins (14). The 24S RNA is probably derived from the 3′ end of the 40S RNA, since it hybridized to a oligo(dT)-primed cDNA probe that had a mean length of 900 nucleotides and a maximum length of about 3,000 nucleotides. This is less than the estimated size of 3,400 nucleotides for the 24S RNA. The size of the 24S RNA indicates a coding capacity of about 120,000 daltons of protein. This is, therefore, sufficient for coding p110. Whether p110 represents the precursor polyprotein of all the structural proteins E1, E2a, E2b, and C has to await comparison of the tryptic peptides of p110 and those of viral proteins. The sum of the molecular weights of the unglycosylated E1 and E2 and the C protein has been estimated to be about 116,000 (14), which is very similar to the molecular weight of p110. The subgenomic 26S mRNA of alphaviruses directs the synthesis of a 130,000-dalton precursor of the structural proteins (9). Both in vivo and in vitro, the *N*-terminally located capsid proteins are cleaved off from the nascent chain during translation. In in vitro-translated free nucleocapsid protein can therefore be detected. In lysates programmed with RV 24S RNA, no free capsid protein has been detected. Thus, if p110 is the precursor of all the RV structural proteins, it must mean that the capsid protein is not cleaved off during translation in vitro.

Single-stranded RNA species, smaller than the genomic RNA, have previously been found in RV-infected cells also by others (7–9, 19, 22). The identity of these RNA species and their relationship to the genomic RNA have remained unclear (5). The results presented here clearly demonstrate the presence of only one subgenomic mRNA in RV-infected cells. It thus appears that the strategy of gene expression of RV (*Rubivirus* genus) is very similar to that of the alphaviruses (9), but different from that of the flaviviruses, viruses comprising two other genera within the *Togaviridae* family (18). Alphaviruses, flaviviruses, and RV have a genome of about the same size. The flaviviruses, however, apparently lack a subgenomic mRNA (26). The structural proteins of the alphaviruses and RV are synthesized as a precursor polyprotein from the subgenomic mRNA, whereas the structural proteins of the flaviviruses may be synthesized individually from the genomic RNA by internal initiation of translation (26).

ACKNOWLEDGMENTS

We thank Annikki Kallio for excellent technical help, Kirsti Tuominen for secretarial help, and Nisse Kalkkinen and Hans Söderlund for helpful discussions and advice.

This work was supported by the Sigrid Jusélius Foundation, the Finnish Cultural Foundation, the Research and Science Foundation of Lääke Oy, and the Oskar Öflund Foundation.

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