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A Transcript from the S Segment of the Germiston Bunyavirus Is Uncapped and Codes for the Nucleoprotein and a Nonstructural Protein

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Analysis of the RNAs present in BHK-21 cells infected with Germiston virus showed that the transcripts from the L and M segments have a size similar to that of their template, whereas two types of complementary RNA are transcribed from the S segment. One, S1, is a full-length "plus" RNA strand (antigenome), and the other, S2, is an incomplete plus RNA strand which serves as mRNA for at least the synthesis of the N protein and a virus-specific nonstructural polypeptide, p12. The 5′ ends of these two transcripts appeared to be identical and complementary to the 3′ ends of the viral RNA. Our results suggest that transcription of the S fragment either stops 100 to 150 nucleotides from the 5′ end of the template, generating an S2 molecule, or continues, generating an S1 molecule. Neither the S1 antigenome nor the S2 mRNA molecules were polyadenylated at their 3′ ends or capped at their 5′ ends.

Bunyaviruses have a segmented, negative single-stranded RNA genome (3), but little is known about their mode of transcription and replication. In vitro RNA polymerase activity has been demonstrated in Lumbo (6) and Uukuniemi (28) virions treated with nonionic detergent, and polysomal RNAs isolated from Lumbo virus-infected cells hybridized to the virion RNA (7).

Genetic and molecular analyses have shown that the small RNA segment (S) codes for the nucleoprotein N and for a nonstructural protein called NS_S (14, 15). The medium-size RNA segment (M) appears to contain the genetic information for at least the two envelope glycoproteins G1 and G2 and for a nonstructural protein, NS_M (14, 16). It is presumed that the third RNA segment (L) codes for the large polypeptide (180,000 to 200,000 daltons) which is associated with the ribonucleoproteins and is believed to be a component of the transcriptase complex. It has been shown that each viral segment is transcribed into a single cRNA molecule (7, 12). This implies that glycoproteins G1 and G2 must be translated from a single polycistronic mRNA derived from the M segment. As support for this concept, Ulmanen et al. (30) have shown that a precursor to the glycoproteins is synthesized in cell-free translation systems. This raised the question of whether a polycistronic mRNA derived from the M RNA segment is translated into a precursor to the three proteins G1, G2, and NS_M or whether two unique monocistronic mRNAs are transcribed from the M RNA segment and are translated into NS_M and precursors to the G1 and G2 proteins, respectively. The same question applies to the S segment and the mRNAs coding for the N and NS_S proteins.

We have reinvestigated this problem using RNA blotting on nitrocellulose and hybridization of ^{32}P-labeled viral probes of high specific activity to characterize the different species of cRNA present in Germiston virus-infected cells. Two types of cRNA were found to be transcribed from the S segment: one is a full-length "plus" RNA strand, which will be designated antigenome, and the other is an incomplete plus strand RNA which serves as mRNA for at least the synthesis of the N protein and a nonstructural polypeptide, p12. Our results suggest that the 5′ ends of these two transcripts are identical and complementary to the 3′ end of the viral RNA. Transcription either stops about 100 nucleotides from the 5′ end of the viral RNA, leading to the synthesis of the mRNA species, or continues, leading to the synthesis of the antigenome. Neither the antigenome nor the mRNA is polyadenylated at its 3′ end or capped at its 5′ end.

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MATERIALS AND METHODS

Cells and virus. Germiston virus was grown in BHK-21 cells in suspension culture. The cells were infected at a multiplicity of infection of 0.01 to 0.001 PFU per cell as previously described (24). Purification of the virus from supernatant fluids was done essentially as previously described (8, 22).

Virus infection and extraction of cytoplasmic RNAs. BHK-21 cell monolayers were infected with Germiston virus at a multiplicity of 20 to 50 PFU per cell. The virus was adsorbed for 1 h at 37°C, and Eagle minimal essential medium supplemented with 5% fetal calf serum was added. Actinomycin D was added 1.75 h after infection, at a final concentration of 5 μg/ml. The cells were labeled from 2 to 6 h postinfection with [^3H]adenosine (10 μCi/ml) or with carrier-free $^{32}P_i$ (0.2 mCi/ml). Cytoplasmic extracts were prepared in reticulocyte standard buffer (RSB) (0.01 M KCl, 0.0015 M $MgCl_2$, 0.01 M Tris hydrochloride, pH 7.4) after disruption by homogenization in a Dounce homogenizer. Cytoplasmic RNAs were extracted with phenol-chloroform (1:1) at pH 9.0.

Isolation and analysis of RNA. RNAs were analyzed by electrophoresis in 2.8% polyacrylamide gels containing 6 M urea after denaturation with glyoxal (21) or in 1% agarose gels containing 5 mM methylmercuric hydroxide by the method of Bailey and Davidson (1). Elution of RNA from the polyacrylamide gels was carried out in 0.5 M LiCl–0.05 M Tris hydrochloride (pH 7.4)–0.002 M EDTA–0.5% sodium dodecyl sulfate. The eluted RNA was precipitated with ethanol and freed of residual acrylamide by sucrose density gradient centrifugation. When necessary, cytoplasmic RNA was centrifuged for 16 h at 32,000 rpm in the Spinco SW41 rotor at 4°C through 15 to 30% sucrose gradients in TE buffer (0.005 M Tris hydrochloride pH 7.4, 0.001 M EDTA).

RNA-RNA hybridization and electrophoresis of double-stranded RNA. Cytoplasmic RNAs were annealed to the virion RNA in the presence of 50% formamide, and the hybrids were treated with RNase T_2 and analyzed by gel electrophoresis as previously described (20).

Blot hybridization of RNA. RNA electrophoresis was carried out on 1% agarose gels containing 5 mM methylmercuric hydroxide. The methylmercury was removed from the gel by washing with 0.5 M ammonium acetate and then with distilled water. The gel was blotted onto a sheet of Gene Screen hybridization transfer membrane (New England Nuclear Corp.) in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate). The membrane was then baked for 2 h at 80°C, prehybridized for 16 h at 42°C in a solution containing 50% formamide, 2× SSC, 0.05 M Na_2HPO_4-NaH_2PO_4, pH 6.5 and 20 μg of yeast tRNA per ml and supplemented with Denhardt solution (0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), and hybridized in fresh solution containing the labeled probe (1×10^6 to 2×10^6 cpm) for 20 h at 42°C. The membranes were washed twice at 60°C for 30 min in 2× SSC, rinsed at room temperature in 0.003 M Tris base, wrapped in Saran wrap, and autoradiographed on Fuji X-ray film at −70°C in the presence of a Du Pont Cronex Lightning-Plus intensifying screen. The probes were prepared by 5′-end labeling of the individual L, M, and S viral segments, partially fragmented by incubation in 50 mM Na_2CO_3 for 1 h at 50°C. After neutralization, the fragments were labeled at their 5′ end with [γ-^{32}P]ATP and polynucleotide kinase.

5′- and 3′-end labeling. RNA was labeled either at its 3′ end with T4 RNA ligase and [^{32}P]pCp or at its 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP as previously described (23). Treatment of RNA with the capping enzyme purified from vaccinia virus (kindly supplied by S. Plotch and R. M. Krug) was carried out before and after β-elimination in the presence of [α-^{32}P]GTP and *S*-adenosyl methionine as already described (10).

In vitro translation. Cell-free translation was carried out for 90 min at 30°C with [^{35}S]methionine in an mRNA-dependent reticulocyte lysate (Amersham Corp.). Cell-free extracts from mouse L cells and wheat germ extracts were prepared and used as previously described (9). Samples were analyzed by electrophoresis on 14% acrylamide–0.093% bisacrylamide gels with the Laemmli buffer system, followed by fluorography (4). Immunoprecipitations were carried out as described (11) with an immune serum prepared from mice inoculated with Germiston virus (kindly supplied by S. Ozden).

Materials. [2,5′,8-^{3}H]adenosine (40 to 60 Ci/mmol), carrier-free $^{32}P_i$ [γ-^{32}P]ATP (3,000 Ci/mmol), [α-^{32}P]GTP (400 Ci/mmol), and L-[^{35}S]methionine (>800 Ci/mmol) were purchased from the Radiochemical Center, Amersham Corp. RNase A was from Worthington Diagnostics, and RNase T_2 and actinomycin D were from Calbiochem-Behring. T4 RNA ligase and 7-methylguanosine 5′-phosphate (pm^{7}G) were purchased from P. L. Biochemicals, Inc., and T4 polynucleotide kinase and calf intestine alkaline phosphatase were from Boehringer Mannheim Biochemicals. Tobacco acid pyrophosphatase was from Bethesda Research Laboratories.

RESULTS

Analysis of the RNAs synthesized in infected BHK-21 cells. Germiston virus-infected BHK-21 cells were exposed to [^{3}H]adenosine or [^{3}H]uridine in the presence of actinomycin D, which does not block the replication of bunyaviruses (5, 31). The time course of synthesis of viral RNAs was determined. Viral RNA synthesis was maximal at 4 to 7 h post infection. The RNAs were extracted from the cytoplasmic extracts of the cells and analyzed by electrophoresis on 2.8% polyacrylamide gels containing 6 M urea (Fig. 1a). Four RNA species were observed, three of which (L, M, and S1) had the same migration rate as the three genomic RNA segments (L, M, and S, respectively). The RNA species S2, which migrated faster than the S segment and was absent from the virion, was the most abundant species found in the infected-cell polysomes (not shown), suggesting that it functioned as mRNA. Its synthesis was detected as early as 2 h and was maximal between 4 and 7 h after infection. The two small RNAs detected in the infected cells were designated S1 and S2 in accordance with the nomenclature already used for the small RNAs found in Uukuniemi virus-infected cells (30). On a methylmercury-agarose gel (Fig. 1b), the four RNA species were also detected, but RNAs in the S region were only occasionally resolved as a doublet. None of these RNAs could be bound to oligodeoxythymidylate-cellulose (data not shown), indicating that the 3′ end of the cRNAs of Germiston virus is not polyadenylated.

The origin of the S2 RNA and the extent of its homology with viral RNAs were determined by hybridization. In vivo ^{32}P-labeled S2 RNA, which was only 7.4% RNase resistant after self-annealing, became 84% resistant when annealed to virion RNA. Moreover, labeled S2 RNA hybridized specifically to the S segment of virion RNA immobilized on a membrane (Fig. 2), showing that RNA S2 is a transcription product derived from the S segment of the genome. In this experiment the S2 RNA used as a probe was isolated from a

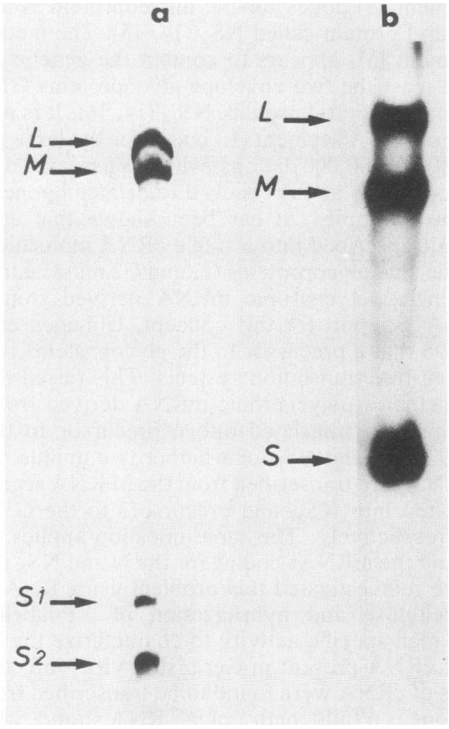

FIG. 1. Analysis of intracellular Germiston virus RNAs. RNA was extracted from infected cells at 6 h after infection and analyzed on (a) a 2.8% polyacrylamide gel containing 6 M urea after denaturation with glyoxal and (b) a 1% agarose gel containing 5 mM methylmercuric hydroxide.

polyacrylamide gel and labeled to high specific activity by in vitro labeling with [γ-^{32}P]ATP and polynucleotide kinase after partial alkaline hydrolysis.

To analyze the viral transcription products, cytoplasmic RNAs were extracted from infected BHK-21 cells at 6 h after infection, separated on a methylmercury hydroxide-containing agarose gel, and transferred onto a membrane. The RNAs on the blot were then hybridized, using as hybridization probes of high specific activity the individual L, M, and S viral RNA segments that had been isolated from a gel and randomly labeled with [γ-^{32}P]ATP and polynucleotide kinase after partial alkaline hydrolysis. Only a single band of RNA hybridized with each probe (Fig. 3). The transcripts derived from the L segment migrated with the same mobility as the L segment (Fig. 3a). Similarly, the transcripts derived from the M (Fig. 3b) and S (Fig. 3c) segments migrated with the mobility of their respective templates. This confirmed previous results (7, 12) indicating that each viral segment is transcribed into a cRNA molecule similar in size to the segment from which it is derived.

Because of the poor resolution between S1 and S2 molecules on methylmercury-containing agarose gels, the cytoplasmic RNAs were also fractionated on a polyacrylamide-urea gel (as in Fig. 1b) and blotted onto a membrane. The randomly labeled S RNA probe hybridized to both S1 and S2 (data not shown), indicating that RNA molecules complementary to the S segment are present in both these RNA species.

Size of the RNA species. To better determine the size of the cRNA transcripts and to discriminate between the intracellular virion-sense RNA molecules and their equal-size RNA molecules (antigenomes), the four intracellular RNA species were isolated and individually self-annealed or annealed with an excess of unlabeled virion RNA. The size of the duplexes was analyzed by electrophoresis on a nondenaturing polyacrylamide gel after RNase T_2 digestion. It should be noted that in this experiment the four intracellular RNAs had been separated on a polyacrylamide-urea gel, but instead of being treated with glyoxal, they were heat-denaturated beforehand. As a consequence, each band of RNA except S2 was contaminated by the smaller molecules.

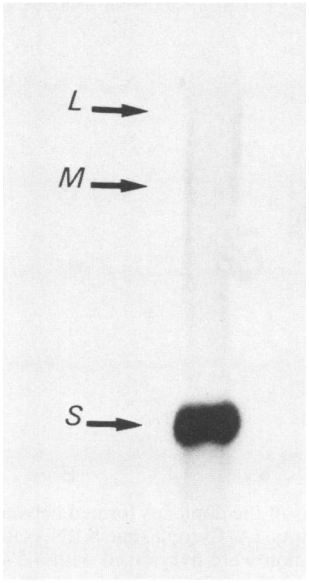

FIG. 2. S2 is transcribed from the S segment of the genome. RNA segments extracted from purified Germiston virus were electrophoresed on 1% methylmercury-agarose gels and blotted onto a transfer membrane. The blot was hybridized with a ^{32}P-labeled S2 RNA probe.

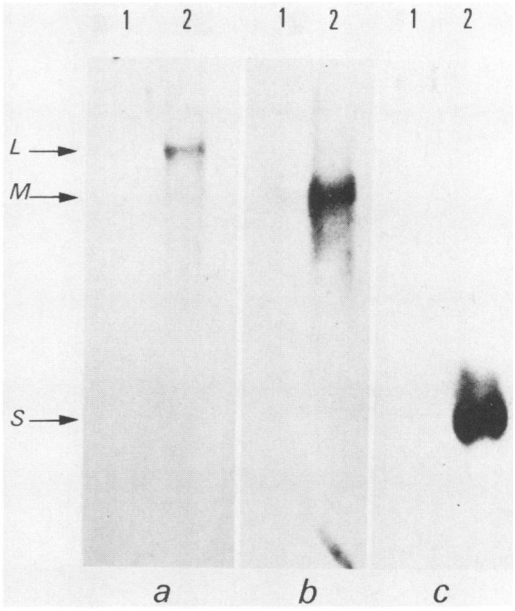

FIG. 3. Analysis of viral RNA transcripts. Cytoplasmic RNA extracted from either mock-infected cells (lanes 1) or Germiston virus-infected cells (lanes 2) was electrophoresed on a methylmercury-agarose gel and transferred to three membranes. The blots were individually probed with the L segment (a), M segment (b), and S segment (c).

Comparison of RNA S1 and S2 showed that only S1 gave rise to RNase-resistant duplexes after self-annealing. After annealing to virion RNA, two duplexes were formed between virion RNA and RNA extracted from the S1 band (Fig. 4, lane 3). The large duplex corresponds to that formed with full-length S1 RNA molecules, and the fast-migrating one corresponds to that formed with S2 RNA (contaminating the S1 band). The duplex formed between virion RNA and S2 RNA (lane 4) migrated like the 950-base pair (bp) *Alu*I fragment of pBR322. By using restriction fragments from pBR322 and bacteriophage lambda DNAs as size markers, it could be estimated that the S2 RNA duplex was about 100 to 150 bp shorter than the full-length S1 RNA duplex, the size of which was about 1,100 bp.

The same experiment was repeated with the hybrids formed with RNAs from bands L (Fig. 4, lane 1) and M (lane 2). Both materials showed 20 to 50% RNase resistance after self-annealing, indicating that they contained molecules of opposite polarity. They yielded RNase-resistant hybrids of the expected (genomic) length after hybridization with excess virion RNA. The same duplexes were observed whether the molecules were self-annealed or annealed with the virion RNA. The sizes of the L and M duplexes were estimated as 7,900 and 5,000 bp, respectively, in a 1% nondenaturing agarose gel. This is in good agreement with the figures previously reported for the molecular weight of the single-stranded RNA segments (23, 24). This type of experiment did not allow us, however, to determine whether the intracellular L and M species contained more than one class of cRNA transcripts, because the resolution of the gels

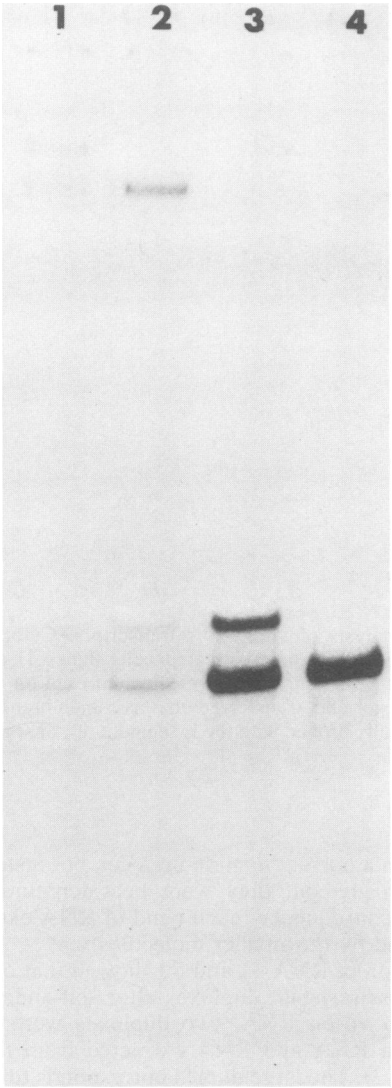

FIG. 4. Analysis of RNA duplexes on a nondenaturing polyacrylamide gel. Cytoplasmic heat-denatured RNA labeled in vivo with ^{32}P was electrophoresed on a denaturing gel. The four bands were eluted separately and hybridized with excess virion RNA. The RNase T_2-resistant hybrids were analyzed on a 3% polyacrylamide gel. Hybrids formed with L RNA (lane 1), M RNA (lane 2), S1 RNA (lane 3) and S2 RNA (lane 4) are shown.

did not allow the detection of a size difference as small as 100 nucleotides in this range of molecular weights.

3′-Terminal sequence of viral S RNA segment transcribed and present in both S1 and S2 transcripts. At least three possibilities could explain why S2 RNA was about 100 nucleotides shorter than the viral S RNA and its full-length transcript S1. (i) Transcription of S2 initiates exactly at the 3′ end of the virion RNA and terminates 100 to 150 nucleotides before the 5′ end of the template; (ii) transcription initiates at the 3′ end of the template, but S2 is generated from S1 by cleavage of a leader RNA and does not retain the 5′-terminal sequences present in S1; or (iii) transcription of S2 is initiated at a site located 100 to 150 nucleotides upstream from the 3′ end of the viral RNA template.

To distinguish between these hypotheses, we first determined whether the 3′ or 5′ end of the viral RNA segment could be protected against RNase digestion after annealing with S2 RNA. Viral S RNA was labeled in vitro at its 3′ end with [^{32}P]pCp and RNA ligase or at its 5′ end with [γ-^{32}P]ATP and polynucleotide kinase after dephosphorylation. The ^{32}P-labeled viral segments were hybridized to tritium-labeled RNA extracted from a cytoplasmic extract and purified by sucrose gradient centrifugation. The fraction of the gradient sedimenting at 12S to 14S was taken as the source of S2 RNA but also contained S1 RNA (Fig. 5A). When the 3′-end-labeled viral S RNA was hybridized with the mixture of S1 and S2 RNAs, the two duplexes formed were labeled. On the other hand, when the 5′-end-labeled viral S RNA was hybridized, the duplex formed with S1 RNA was labeled, whereas that formed with S2 was not.

This experiment demonstrated clearly that the sequence of the 3′ end of the virion RNA is transcribed and present in S1 and S2 RNAs and that transcription either continues to the end of the template, generating a molecule of S1 RNA, or terminates before the 5′ end of the template, generating a molecule of S2 RNA.

S2 RNA uncapped at the 5′ terminus. Several data support the evidence that S2 but not S1 RNA functions as an mRNA. Cytoplasmic extracts were fractionated into polysomal and nucleocapsid fractions on either sucrose (7) or cesium chloride gradients (25). Analysis of the RNA species present in these fractions indicated that S1 RNA was associated with the nucleocapsids but was not found in the polysomal fraction (data not shown). The RNAs from the polysomes were resolved by electrophoresis into three bands: RNA S2 and the transcription products derived from the L and M segments, the sizes of which were similar or identical to

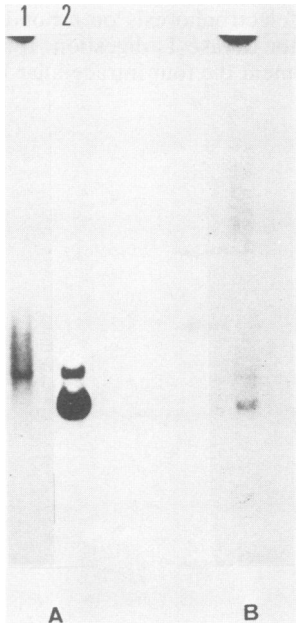

FIG. 5. Analysis of the duplexes formed between viral RNA and the S RNA transcripts. (A) Cytoplasmic S RNAs isolated by sucrose gradient centrifugation were hybridized with a 5′-end-labeled (lane 1) or 3′-end-labeled (lane 2) S RNA segment, and the RNase-resistant duplexes were electrophoresed on 3% polyacrylamide gels. (B) Cytoplasmic S RNAs were labeled with the vaccinia virus capping enzyme in the presence of [α-^{32}P]GTP and S-adenosyl methionine and hybridized to virion RNA. RNase-resistant duplexes were analyzed on a 3% polyacrylamide gel.

those of their respective templates. These results suggest that S1 RNA must be located in positive-strand nucleocapsids containing the two internal proteins L and N, which are associated with RNA polymerase activity. This structure probably functions as a replication complex for the synthesis of progeny virion RNA.

In an attempt to analyze the 5′ end of S1 and S2 RNAs, we looked for the presence of cap structures or di- or triphosphate nucleotides. Direct analysis of RNAs uniformly labeled with ^{32}P did not give a conclusive answer. We therefore took advantage of the capping enzyme isolated from vaccinia virus, which specifically labels the 5′ end of di- or triphosphate-terminated RNAs in the presence of [α-^{32}P]GTP and *S*-adenosyl methionine, to try to label the 5′ end of RNA S2. As a control, we ran, in a parallel reaction, an RNA sample that had been chemically decapped by periodate oxidation followed by β elimination.

RNAs S1 and S2 were purified from a cytoplasmic extract by sucrose gradient centrifugation as in the earlier experiment. Because this fraction also contained cellular RNAs which could be labeled by the capping enzyme, we then annealed the sample with excess cold virion RNAs and analyzed the resulting RNase T_2-resistant duplexes by polyacrylamide gel electrophoresis (Fig. 5B).

Analysis of the RNase-resistant duplex molecules showed that S2 as well as S1 RNA was labeled without prior decapping treatment (Fig. 5B), indicating that both transcripts were naturally uncapped. Labeling occurred to about the same extent when the RNAs were chemically decapped before the capping reaction (data not shown).

The digestion was performed under the conditions originally used to analyze the duplexes formed between influenza virus RNAs and their transcription products (i.e., 20 U of RNase T_2 per ml for 2 h at 37°C) (20); this treatment trims the 5′ capped, host-derived sequence of the mRNA, as well as any unpaired region in the molecule.

If, therefore, capped or uncapped host-derived sequences existed at the 5′ end of bunyavirus mRNA (2), they should have been eliminated, and this type of mRNA would not have been detected. It should also be noted that the cytoplasmic RNAs used for this experiment also contained the S genomic segment, which terminates with a 5′ di- or triphosphate and could have been capped (data not shown). However, because hybridization was performed with an excess of cold viral RNA, it is likely that the label in the S1 duplex was associated with the antigenome only. This problem does not apply to RNA S2, since the sequence complementary to the 5′ end of the viral RNA is not conserved in the duplexes formed with S2 RNA (Fig. 5A).

S2 RNA codes for polypeptides N and p12. To show that S2 RNA functioned as an mRNA, the in vitro translation of S2 was studied in a cell-free system prepared from rabbit reticulocytes or L cells. The S2 translation products were examined in 14% polyacrylamide gels and compared with the proteins synthesized in Germiston virus-infeced cells. Analysis of the latter revealed the existence of at least three small virus-induced nonstructural polypeptides with molecular weights of 12,000 (p12), 16,000 (p16), and 20,000 (p20) in addition to the four known structural proteins L, G1, G2, and N (Fig. 6A). At least two of these nonstructural polypeptides, p12 and p16, did not migrate at the same rate as any of the labeled polypeptides present in mock-infected cells.

When added to the rabbit lysate or to the L-cell extract, RNA S2 directed the synthesis of two proteins that comigrated with nucleoprotein N and with the nonstructural polypeptide p12, respectively (Fig. 6B). RNA extracted from noninfected cells directed the synthesis of several polypeptides, but none of them comigrated with N or p12. Both the N protein synthesized in vitro and that labeled in vivo could be immunoprecipitated with an antivirion immune serum, but the same serum failed to immunoprecipitate polypeptide p12 whether it was synthesized in vitro or in infected cells (data not shown). Preliminary data on tryptic peptide analysis suggest that the two polypeptides synthesized in vitro are authentic N and p12 proteins.

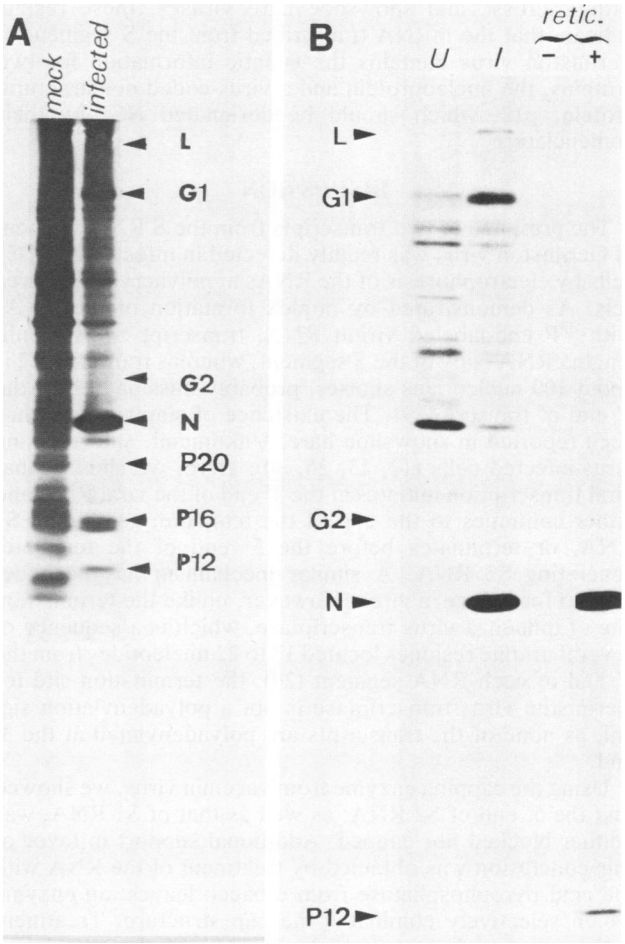

FIG. 6. Analysis of viral polypeptides. (A) Proteins of infected or mock-infected BHK-21 cells were labeled with [^{35}S]methionine and analyzed as described in the text. (B) RNA S2 fractionated in a polyacrylamide gel was translated in a rabbit reticulocyte lysate cell-free system, and the proteins were analyzed. Symbols: +, S2 RNA added; −, no RNA added. Infected (I) and uninfected (U) cell extracts were run as markers.

Viral sequences have been cloned into bacteriophage M13. One clone, which will be described elsewhere, contains an insert of about 500 nucleotides corresponding to virion-sense sequences of the S segment. When RNAs from infected cells were selected by hybridization with the DNA of this recombinant M13 bacteriophage immobilized on nitrocellulose and then used to direct cell-free protein synthesis in an in vitro L-cell extract, the two polypeptides N and p12 were again synthesized (manuscript in preparation). Confirming the results obtained by Fuller and Bishop (14)

with Lacrosse and snowshoe hare viruses, these results indicate that the mRNA transcribed from the S segment of Germiston virus contains the genetic information for two proteins, the nucleoprotein and a virus-coded nonstructural protein, p12, which should be designated NS_S by their nomenclature.

DISCUSSION

The presence of two transcripts from the S RNA segment of Germiston virus was readily detected in infected BHK-21 cells by electrophoresis of the RNAs in polyacrylamide-urea gels. As demonstrated by duplex formation of the RNAs with ^{32}P end-labeled virion RNA, transcript S1 is a full-length cRNA copy of the S segment, whereas transcript S2 is about 100 nucleotides shorter, probably missing part of the 3′ end of transcript S1. The existence of similar RNAs has been reported in snowshoe hare, Uukuniemi, and Akabane virus-infected cells (12, 13, 26, 30). Here, we showed that viral transcription initiates at the 3′ end of the viral RNA and either continues to the end of the template, generating S1 RNA, or terminates before the 5′ end of the template, generating S2 RNA. A similar mechanism has been described for influenza virus. However, unlike the termination site of influenza virus transcriptase, which is a sequence of several uridine residues located 17 to 22 nucleotides from the 5′ end of each RNA segment (29), the termination site for Germiston virus transcriptase is not a polyadenylation signal, as none of the transcripts are polyadenylated at the 3′ end.

Using the capping enzyme from vaccinia virus, we showed that the 5′ end of S2 RNA, as well as that of S1 RNA, was neither blocked nor capped. Additional support in favor of this conclusion was obtained by treatment of the RNA with the acid pyrophosphatase from tobacco leaves, an enzyme which selectively eliminates the cap structure. Treatment with pyrophosphatase abolished 83.6% of the translation activity of globin mRNA, but only 28% of that of S2 RNA, which in fact could be due to partial nonspecific degradation of the RNA (data not shown).

The full-length cRNA of the S segment, RNA S1, appears to be a good candidate for a replicative template. This RNA was never found associated with polyribosomes, but only with nucleocapsids. Such positive-strand nucleocapsids must contain at least the two internal proteins N and L. The encapsidation of S1 RNA, which has also been observed for other bunyaviruses (7, 30) raises the question of why S1 RNA is encapsidated whereas S2 RNA is not. A possible answer could be that encapsidation of the RNA starts from a recognition sequence located at the 3′ end of the molecule corresponding to a binding site for the N polypeptide. This site, which might be the panhandle or part of it (23), would also be found at the 3′ end of the genome or the antigenome, as the 3′ and 5′ ends of the RNA are complementary to each other, but would be absent from S2, as S2 lacks the 3′ end of S1.

The only feature which distinguishes S2 from S1 molecules is the sequence of 100 to 150 nucleotides located at the 3′ end of S1. The existence of two types of positive transcripts, mRNA and antigenome, has been well documented for several families of negative-strand RNA viruses, both nonsegmented and segmented (such as myxoviruses). The mechanisms involved in the synthesis of the mRNAs and antigenome RNAs of influenza virus appear to be different. Transcription of the polyadenylated mRNA is initiated by host cell primers which donate their cap and first 10 to 14 nucleotides to the viral mRNA (9, 20, 27). Transcription of the antigenome is probably initiated without primers at the exact 3′ end of the genome, as suggested by the presence of a 5′ di- or triphosphate end (17). The presence of di- or triphosphate nucleotides at the 5′ end of Germiston virus mRNA and antigenome RNA suggests that initiation of transcription of the two types of cRNA is carried out by the same or very similar mechanisms. However, the strategy we used to demonstrate the absence of a cap structure in RNA S2 implies that any sequence not derived from the virion RNA would have been eliminated during digestion of the duplex molecules with RNase T_2. Therefore, we cannot exclude the possibility that these uncapped mRNAs might represent a minor population among others primed by host cellular RNAs, as very recently published by Bishop et al. (2).

In vitro translation directed by S2 RNA in cell-free systems led to the synthesis of two proteins, N and p12. This was unexpected, as it is believed that translation of eucaryotic mRNAs begins at the 5′-proximal AUG initiation codon or, at least, at the 5′-uttermost $\overset{A}{G}$XXAUGG sequence (18, 19). Germiston virus S2 RNA may therefore be one of the few exceptions violating the rule of the scanning model. However, we cannot eliminate the possibility that the two polypeptides N and p12 might be translated from two different species of mRNA that comigrated in the gel. These mRNAs would be colinear except for their 5′ sequences, each containing as the first 5′-proximal AUG triplet the initiation codon that precedes the open reading frame coding for a given polypeptide, as recently described by Patterson et al. (25). It is also possible that in the cell-free systems we used (reticulocyte lysate and L-cell extracts), RNA S2 was cleaved, generating fragments in which internal AUGs became exposed as 5′-proximal initiation codons. Nucleotide sequencing of the S segment, which is presently in progress, should help resolve these problems.

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