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Analyses of the mRNA Transcription Processes of Snowshoe Hare Bunyavirus S and M RNA Species

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The time course of synthesis of snowshoe hare bunyavirus small (S)- and medium (M)-sized viral RNA (vRNA), viral cRNA (vcRNA), and mRNA species was analyzed by using single-stranded DNA probes representing the S- and M-coded gene products. In the presence of puromycin, an inhibitor of protein synthesis, the subgenomic S mRNA species were detected, but not full-length S vcRNA or S vRNA species. No M-related RNA species were identified in puromycin-treated cells. In the absence of puromycin, full-length M and S vRNA, S vcRNA, and subgenomic S mRNA species were observed, as well as apparently full-length M vcRNA species, presumably including the approximately similar-sized M mRNA species. The 5′ ends of the S and M mRNA species have been shown to be heterogeneous and some 12 to 17 bases longer than the ends of their corresponding presumptive replicative vcRNA species, in agreement with an earlier report that they represent nonviral primer sequences (D. H. L. Bishop, M. E. Gay, and Y. Matsuoko, Nucleic Acids Res. 11:6409–6418, 1983). The 3′ ends of the M and S mRNA species were found to be shorter by some 60 and 100 nucleotides, respectively, than those of their corresponding full-length vcRNA species. Comparison of the 3′ noncoding regions of the S and M vcRNA species revealed that there are conserved sequences following the translation termination codons of the two RNA species. One of these conserved sequences is a pyrimidine-rich template sequence that is approximately 20 nucleotides beyond the deduced S mRNA transcription termination site.

Bunyaviruses are enveloped, negative-sense, RNA viruses that have a tripartite, single-stranded, RNA genome (5, 8). Molecular and genetic studies have established that the bunyavirus small-sized (S) RNA species codes for two gene products, a nucleoprotein, N, and a nonstructural protein, NS_S (2, 7, 13, 17, 18, 20). These two proteins are read from overlapping reading frames in the viral-complementary sequence (17). So far (6, 12, 29; this study), only a single S mRNA species has been identified, although claims were made for multiple S mRNA initiations for the related La Crosse (LAC) bunyavirus (27). The snowshoe hare (SSH) medium-sized (M) RNA species codes for three proteins, the two viral glycoproteins, G1 and G2, and a second nonstructural protein, NS_M (16, 21). DNA cloning studies have shown that there is a single, continuous, open reading frame in the M viral-complementary sequence (16). The third viral RNA (vRNA) species, L, is believed to code for a large protein present in virions and in virus-infected cells that may be a component of the transcriptase and replicase of the virus (8).

Analyses of SSH and other bunyavirus S mRNA species have established that they are shorter than the S vRNA species (10, 13, 29, 30). The 5′ proximal sequence of the S mRNA species is complementary to the 3′ end of the S vRNA species (6, 29). In addition, it contains 5′-terminal, nonviral, sequences (6, 29). This was first shown by using a viral-sense oligonucleotide primer (complementary to an internal mRNA sequence) to transcribe and then clone the 5′ ends of the S viral cRNA (vcRNA) species (6). From such analyses two types of transcripts were characterized. One was essentially an exact complementary copy of the 3′ end of the vRNA and presumably represented an S RNA viral-complementary replicative intermediate. The other was longer, with additional, heterogeneous, 5′ end sequences extending some 13 to 14 nucleotides beyond the end of the vcRNA. These data have been interpreted to indicate that the longer transcripts represent mRNA species, analogous to the influenza virus primer-initiated mRNA species (11, 24, 31). No evidence for different mRNA species representing the individual N and NS_S gene products was obtained in our previous analyses (6), although because only a few DNA clones were analyzed, a second S mRNA species may not have been represented. To investigate the question of whether multiple S mRNA species can be detected, an alternative approach was used, back copying the ends of the population of S mRNA species with a 5′-labeled primer. The labeled products were recovered and sequenced directly. The results obtained again provided evidence for a single, heterogeneous primer-initiated mRNA species as well as a shorter, exact copy, presumptive replicative intermediate RNA species. Equivalent data are also reported in this communication for the M RNA species. While these studies were in progress, similar results were described by Patterson and Kolakofsky for the related LAC S mRNA species (29). Analyses of the 3′ ends of the SSH S and M mRNA species by hybridizing end-labeled viral-sense DNA species to the respective mRNA species and recovering the trimmed labeled duplexes by S1 nuclease digestion provided evidence for the sites of termination of S and M mRNA transcription.

Previously, by using sensitive hybridization analyses, Vezza et al. reported that bunyavirus mRNA species are synthesized in infected cells in the presence (primary transcription) and absence (primary plus secondary transcription) of inhibitors of protein synthesis (33). However, two recent studies failed to detect mRNA synthesis in infected

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cells in which mRNA translation is inhibited (1, 29), despite the observations that bunyaviruses and uukuniemi viruses have a virion transcriptase (9, 32). We therefore reinvestigated this question in a time course analysis with DNA probes to detect viral and vcRNA (replicative mRNA species). The results confirmed our previous studies and indicate that like other negative-sense RNA viruses, bunyaviruses can direct the synthesis of mRNA species in cells in which protein synthesis is inhibited.

MATERIALS AND METHODS

Cells and virus. The origin of the SSH virus used for these studies has been described previously (14). Virus was grown in monolayers of BHK-21 cells in Eagle medium containing 10% newborn calf serum and purified as described elsewhere (26).

RNA extraction. RNA was recovered from purified virus by extraction with a phenol-8-hydroxyquinoline-*m* cresol-chloroform mixture (22) and recovered by alcohol precipitation. After further alcohol precipitations, the RNA was suspended in sterile water and frozen at −70°C. For analyses of the course of RNA synthesis in infected cells, 0.1-ml portions of purified virus suspended in Eagle medium were used to infect 4×10^5 confluent BHK-21 cells at a multiplicity of approximately 50 PFU per cell. After absorption at 18°C for 30 min, the excess inocula were removed and the cultures were overlaid with 5-ml volumes of Eagle medium containing 10% newborn calf serum. After 1 h of incubation at 33°C, the media were replaced to remove desorbed virus and the incubations continued to the indicated times. For some of the analyses, puromycin (100 μg per ml) was added to the culture medium for 1 h before virus infection as well as during incubation of the cells. Cells were washed in phosphate-buffered saline, and the RNA was recovered as described elsewhere (13). RNA preparations were resolved by gel electrophoresis in the presence of methyl mercury (4) and prepared for blotting as described by Alwine and associates (3) by using a Gene-Screen (New England Nuclear Corp., Boston, Mass.). After blotting, the membranes were air dried, baked at 80°C for 2 h, and hybridized for 16 h at 42°C as described by Denhardt (15) with ^{32}P-labeled, individual viral-sense or viral cDNA (vcDNA) strands representing coding regions of SSH S or M DNA (7, 16). Membranes were washed and autoradiographed.

Oligonucleotides synthesis. An oligodeoxyribonucleotide representing M RNA residues 70 to 92 (16; i.e., 5′ TGACTGCAAACAGTATCAATATG) was synthesized by a solid-phase phosphotriester method and purified by high-pressure liquid chromatography (19, 34). It was phosphorylated at its 5′ terminus by polynucleotide kinase with 7,000 Ci/mmol of [γ-^{32}P]ATP as described by Ihara and associates (23) before use for cDNA synthesis. Labeled products were recovered and sequenced by the Maxam and Gilbert method (25).

RESULTS

Analyses of the 5′ ends of populations of SSH S mRNA and vcRNA species. In previous studies (6), a 29-nucleotide-long viral-sense oligonucleotide representing residues 121 to 149 of the SSH S RNA sequence (from the 3′ end, i.e., at the beginning of the N and NS_S coding regions; see reference 7) was used to back copy the 5′ ends of gradient-resolved SSH mRNA and presumptive replicative intermediate S vcRNA species. Two transcript-sized classes were identified (ca. 149 and ca. 164 nucleotides long). The transcripts were recovered, tailed with oligo(dA), back copied with oligo(dT), and cloned into pBR322. Several independent clones were sequenced, providing results which indicated that, as expected for replicative intermediate RNA species, the ca. 149-nucleotide-long transcripts were exact copies of the S RNA that extended to the penultimate nucleotides of the vRNA. The ca. 164-nucleotide-long transcripts were also found to be exact copies of the viral sequence up to the terminal (or penultimate nucleotide), with an additional extension of some 13 to 14 nucleotides that varied in sequence from clone to clone. These data were interpreted to indicate that the SSH S mRNA species had 5′ non-vRNA sequences which, by analogy with influenza virus (11, 24, 31), represented cellular derived primer sequences that had been used for viral mRNA synthesis. Because only a small number of clones of cDNA were analyzed, the question remained whether there were other species of S mRNA, e.g., representing NS_S mRNA species. This question has now been addressed by specifically labeling the oligonucleotide primer

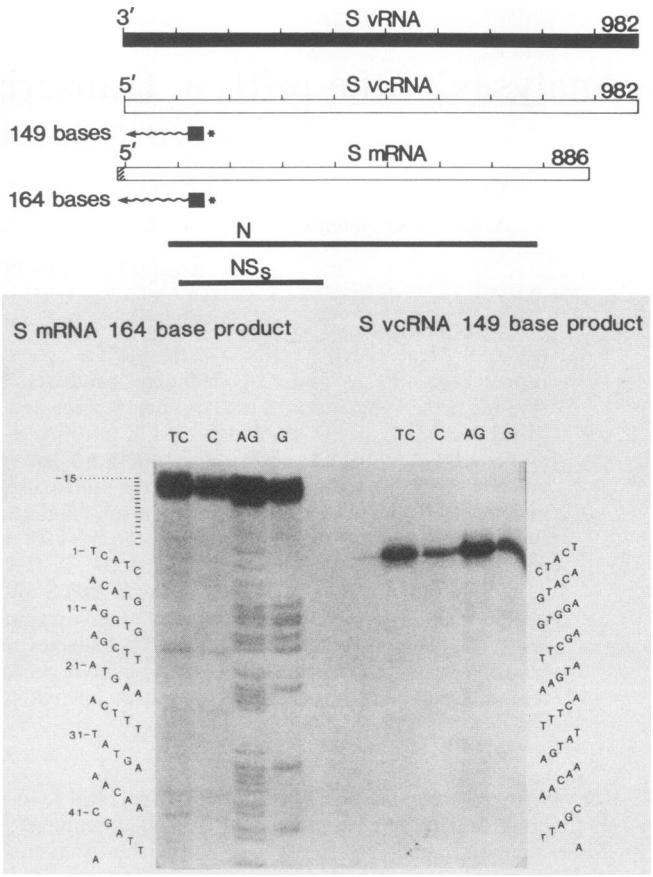
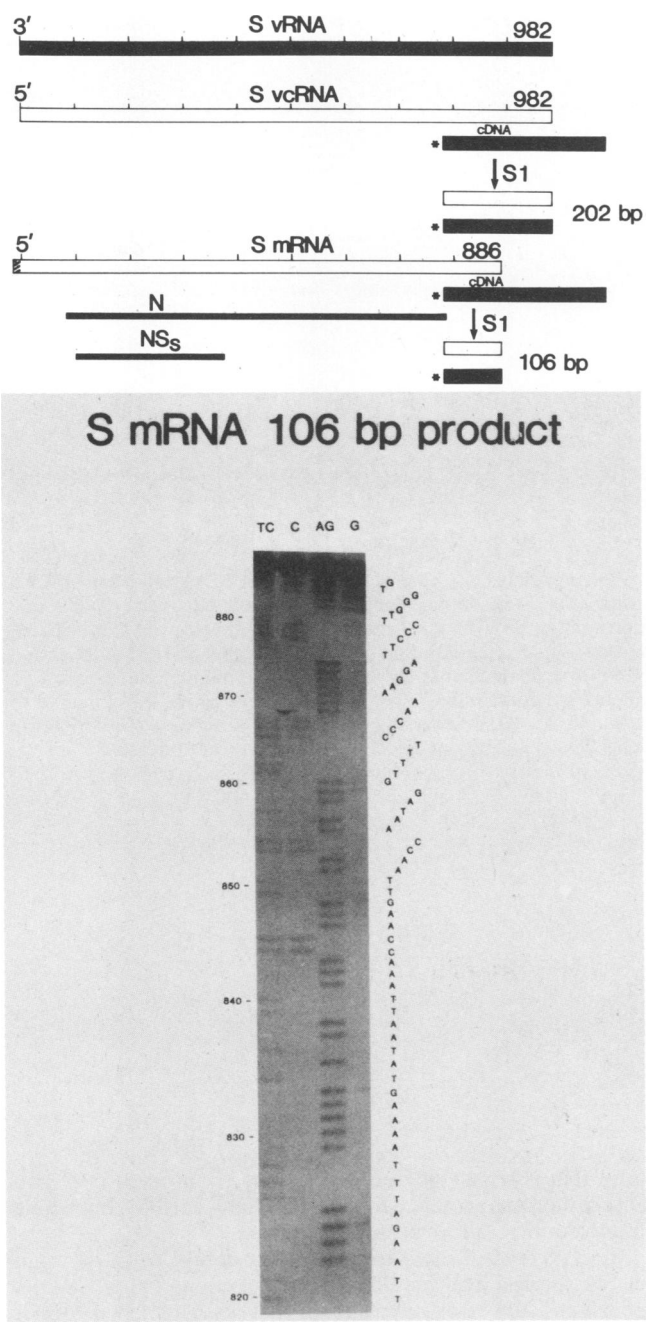


FIG. 1. Analyses of the 5′ ends of SSH S vcRNA and mRNA species. Total cytoplasmic RNA extracted from SSH virus-infected cells 10 h postinfection (multiplicity of infection, 10) was resolved by centrifugation in gradients of sucrose, and the fractions containing S viral and mRNA species were identified by Northern hybridization and in vitro translation with rabbit reticulocyte lysates (6). The fractions were pooled and used to template cDNA synthesis with four unlabeled deoxyribonucleoside triphosphates, reverse transcriptase, and a 5′-labeled 29-residue-long synthetic oligonucleotide primer representing S vRNA residues 121 to 149 (6, 23; schematic). As described previously (6), after electrophoresis in 6% gels containing 7 M urea, two labeled cDNA bands were obtained (ca. 149 and ca. 164 nucleotides long). The bands were recovered and sequenced by the Maxam and Gilbert method (25).



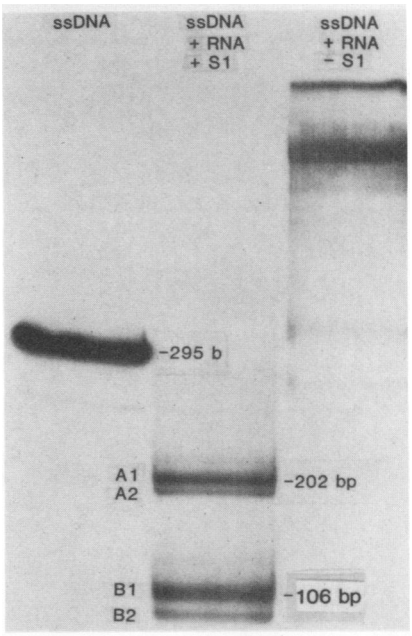
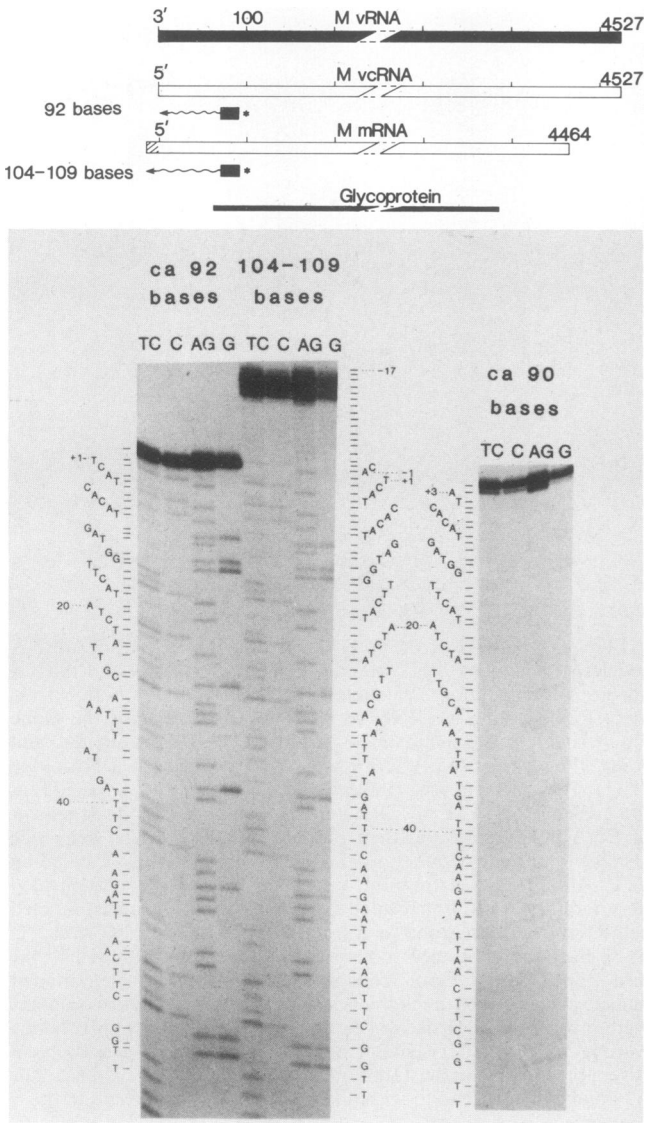


FIG. 2. Analyses of the 3′ ends of SSH S vcRNA and mRNA species. To identify the 3′ termini of SSH S vcRNA and mRNA species (schematic), a 295-nucleotide-long *Sfa*NI restriction fragment [representing S vRNA residue 781 to the end of the clone (approximately 200 nucleotides), a poly(A) cloning sequence, and some 80 base pairs of pBr322] was derived from SSH S DNA clone 17 (7). The fragment was labeled at its 3′ termini by [α-^{32}P]TTP in the presence of dATP, dCTP, and dGTP and the Klenow fragment of DNA polymerase and then purified. Because of the sequence specificity of the enzyme, only the viral-sense DNA was labeled by TTP. After heat denaturation, the DNA was either self-annealed (top right, left tract) or annealed for 18 h to total SSH virus-infected cell RNA (right tract) and then treated for 10 min at 18°C with 5 U of S1 nuclease in 30 mM sodium acetate (pH 4.6)–50 mM NaCl–1 mM $ZnSO_4$–5% glycerol (center tract) to digest single-stranded nucleic acids. The S1 nuclease-resistant products and other samples were resolved in a 5% native gel, and the A1, A2, B1, and B2 bands were recovered. After sequencing, only the 106-base-pair B1 band gave a presentable pattern (bottom left); however, the 202-base-pair A1 band could be read to within five nucleotides of the end of the S sequence.

at its 5′ terminus and using it in back-copy reactions with unlabeled deoxyribonucleoside triphosphate precursors, reverse transcriptase, and gradient-resolved S mRNA and vcRNA species (Fig. 1). As shown previously (6) and exemplified in the schematic in Fig. 1, two types of transcript were obtained (ca. 149 and ca. 164 nucleotides long). They were recovered and sequenced. Although present in small quantities, the ca. 149-nucleotide-long transcript gave a sequence that appeared to be an exact copy of the terminal 3′ nucleotides of the S RNA (Fig. 1). The ca. 164-nucleotide-long transcript was a copy up to the 3′ end of S, with an additional heterogeneous sequence of some 12 to 15 nucleotides including the suggestion of an A residue at the −1 position. These results confirmed previous studies which indicated that the SSH S mRNA species have heterogeneous, nonviral, 5′ termini. While these studies were in progress, similar results were reported by Patterson and Kolakofsky for LAC S RNA species (29).

Analyses of the 3′ ends of populations of SSH S mRNA and vcRNA species. It has been shown that overall, despite the additional 5′ sequence on the mRNA species, bunyavirus S mRNA species are smaller than the S vRNA species (10, 13, 29, 30). This observation suggests that transcription of bunyavirus S mRNA is terminated before the end of the template S RNA is reached. To identify the approximate site of mRNA termination, a 295-base-pair *Sfa*NI restriction fragment was derived from SSH S DNA clone 17 [7; representing SSH S DNA residues 781 to the end of the clone plus



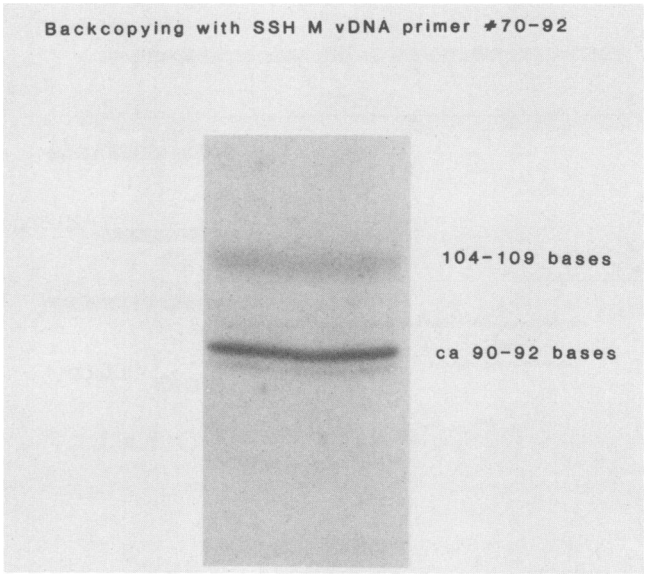


FIG. 3. Analyses of the 5′ ends of SSH M vcRNA and mRNA species. A 23-nucleotide-long synthetic oligodeoxyribonucleotide representing SSH M viral residues 70 to 92 (16) was synthesized, labeled at its 5′ end (23), and used to back copy SSH M vcRNA and vRNA species (schematic). After denaturation and electrophoresis in a 6% gel containing 7 M urea, radioactive products estimated to be ca. 90, ca. 92, and ca. 104 to 109 bases long were identified (top right). They were recovered and sequenced (bottom left).

a poly(A) cloning sequence and approximately 80 base pairs of the vector pBr322 sequence]. The fragment was labeled at its 3′ termini with [α-^{32}P]TTP in the presence of unlabeled dATP, dCTP, and dGTP plus the Klenow fragment of DNA polymerase. The labeled fragment was recovered, heat denatured, and annealed to SSH virus-infected cell RNA (Fig. 2). The products were treated with S1 nuclease, and two major (A1, B1) and two minor (A2, B2) duplexes were resolved by gel electrophoresis (Fig. 2). Too few counts of the minor products were recovered for further analysis. The sequence of the major ca. 106-base-pair product (B1) could be read from SSH S RNA residue 781 to residue 886 and appeared to extend an additional two or three residues. The identities of the terminal nucleotides were not clear (Fig. 2). Although only small amounts of the ca. 202-base-pair product (A1) were recovered, the sequence could be read from residue 781 to within five residues of the 5′ end of the S RNA sequence (data not shown).

We concluded that two S vcRNA species were present in the infected cell extracts, an approximately full-length vcRNA and a shorter species that is presumed to be the subgenomic S mRNA species. The latter represents a transcript that is some 100 nucleotides shorter at its 3′ end than the presumptive replicative intermediate vcRNA but some 15 nucleotides longer at its 5′ end.

Analyses of the 5′ ends of populations of SSH M mRNA and vcRNA species. The results of DNA cloning of the M RNA species of SSH virus have shown that a single gene product is coded in the viral-complementary sequence (16). Other than the time course of synthesis of the individual types of virus-induced RNA species (33), no studies of the character of the M mRNA and M replicative intermediate RNA species have been reported. To determine whether SSH M mRNA species have heterogeneous, nonviral, 5′ end sequences analogous to those identified for bunyavirus S mRNA species, a viral-sense primer was synthesized representing a sequence proximal to the 3′ end of the M RNA (16; i.e., residues 70 to 92). The primer was phosphorylated at its 5′ terminus with [γ-^{32}P]ATP and used to back copy M vcRNA species (Fig. 3). Three sizes of transcript were made (ca. 90, ca. 92, and ca. 104 to 109 nucleotides long). Each was recovered and sequenced (Fig. 3). The ca. 90- and ca. 92-nucleotide-long transcripts were exact copies to the +3 and +1 nucleotides of the 3′ end of the M vRNA, and each

had an additional final nucleotide that could not be identified. It is not known whether the terminal nucleotide represents a true transcript of an end nucleotide or whether it is an artifact of the reverse transcriptase reaction. The diffuse character of the ca. 104- to 109-nucleotide transcript indicated that it was a mixture of products. The sequencing results confirmed this observation and showed that it was an exact copy to the 3′ end of the M RNA with, additionally, some 12 to 17 nucleotides that were heterogeneous in sequence but with an indication of a preference for A at the −1 and C at the −2 position. It was concluded from these studies that two types of vcRNA were present in the cell extracts: one was a transcript from the 3′ end of the vRNA, and the other had additional 5′ heterogeneous sequences, similar to the putative primer sequences identified for the S mRNA species.

Analyses of the 3′ ends of populations of SSH M mRNA species. To determine whether transcription of bunyavirus M mRNA is terminated before the end of the template M RNA is reached, a 450-base-pair *Dde*I restriction fragment was derived from SSH M DNA clone 72-114 [16; representing SSH M DNA residue 4340 to the end of the clone plus a poly(C) cloning sequence and approximately 250 base pairs of the vector pBR322 sequence]. The fragment was labeled at its 3′ termini with [α-^{32}P]TTP in the presence of unlabeled dATP, dCTP, and dGTP by using the Klenow fragment of DNA polymerase. The labeled fragment was recovered, heat denatured, and annealed to SSH virus-infected cell RNA (Fig. 4). The products were treated with S1 nuclease, and a single major ca. 125-base-pair duplex was resolved by gel electrophoresis (Fig. 4). Too few counts of the product were recovered for further analysis. No band equivalent to the expected ca. 190-base-pair duplex representing replicative M vcRNA species was observed. The reason is not known; it may have been due to a much lower abundancy of the full-length M vcRNA species relative to the M mRNA species.

Analyses of the synthesis of viral-induced S and M RNA species in infected cells in the presence or absence of inhibitors of protein synthesis. In a previous study (33), labeled vRNA and liquid hybridization were used to investigate the synthesis of vcRNA in cells infected with SSH temperature-sensitive mutants and wild-type viruses. The results indicated that neither actinomycin D, α-amanitin, nor rifampin significantly inhibited vcRNA synthesis, although there were (approximately) 1-log reductions in virus yields when actinomycin D or rifampin was used. In comparison with untreated infected cells, when cycloheximide or puromycin was used to inhibit protein synthesis, small amounts of vcRNA were identified in both wild-type and mutant virus-infected cells incubated at permissive temperatures. In molar terms, with puromycin and individual S, M, or L vRNA species as hybridization probes, some 20 times more S vcRNA than M or L RNA species was detected by 8 h postinfection (33). These analyses did not discriminate between replicative and mRNA species in the infected cell extracts. Recently, two laboratories reported that they were not able to detect bunyavirus mRNA in infected cells that were incubated in the presence of protein synthesis inhibitors (1, 29), despite the fact that one of these laboratories has reported the existence of a transcriptase activity associated with virus preparations (28), in concert with previous reports of bunyavirus and uukuniemi virus virion transcriptases (9, 32). In view of these discrepancies, we analyzed the course of synthesis of M and S viral and vcRNA species in SSH virus-infected cells incubated in the presence or absence of inhibitors of protein synthesis by using single-stranded DNA probes representing, respectively, viral-complementary and viral sequences.

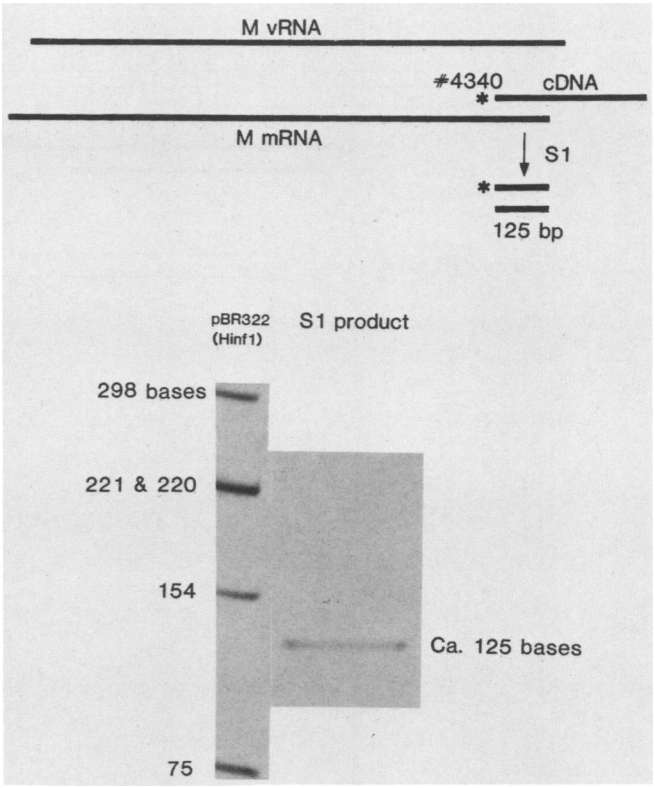
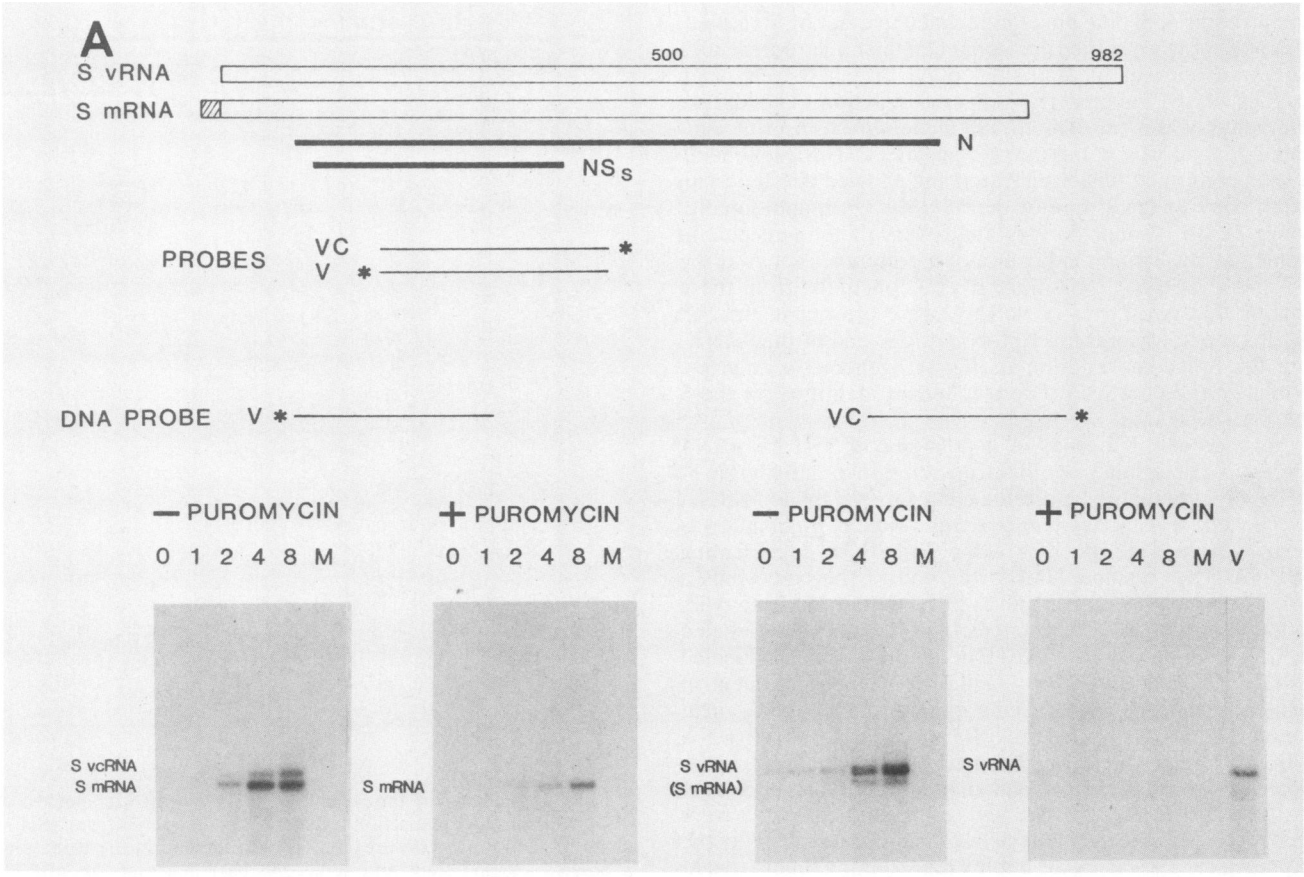


FIG. 4. Analysis of the 3′ end of SSH M mRNA species. A 450-base-pair *Dde*I restriction fragment was recovered from SSH M clone 72-114 [16; M DNA residue 4340 to the end of the clone plus a poly(C) cloning tract and some 250 base pairs of the pBR322 sequence]. The DNA was labeled at its 3′ termini with [α-^{32}P]TTP, heat denatured, and annealed to SSH virus-infected cell RNA. After S1 nuclease digestion (Fig. 2), a ca. 125-base-pair product was obtained (bottom panel). Insufficient counts were recovered to allow sequence analyses.

To identify S RNA species in infected cell extracts, we recovered a 243-base-pair *Hin*fI restriction fragment from SSH S clone 17 (7; representing residues 173 to 464, i.e., encompassing the N and NS_S genes). After being labeled at its 3′ termini with [α-^{32}P]dATP, nonradioactive dCTP, TTP, and dGTP, and the Klenow fragment of DNA polymerase, the restriction fragment was isolated and heat denatured, and the individual strands were recovered after resolution by gel electrophoresis. Because the two DNA strands were separated by only some 4 mm, when samples were rerun for purity monitoring, a small amount of contamination of the vcDNA probe by viral DNA (vDNA) was observed, although the vDNA probe was judged to be free from vcDNA contamination. The sequences of the two strands were known from prior analyses (6). Each was used to identify (by Northern hybridization) the respective cRNA species in extracts of infected cells that had been resolved by agarose gel electrophoresis in the presence of methyl mercury (Fig. 5A). Thus, the vDNA probe was used to identify S vcRNA and mRNA species, and the vcDNA probe was used to anneal to S vRNA species. As expected, no vRNA species were found in mock-infected cell extracts (M lanes, Fig. 5A). As a function of the infected cell incubation time (0, 1, 2, 4, 8 h), increasing quantities of subgenomic S mRNA species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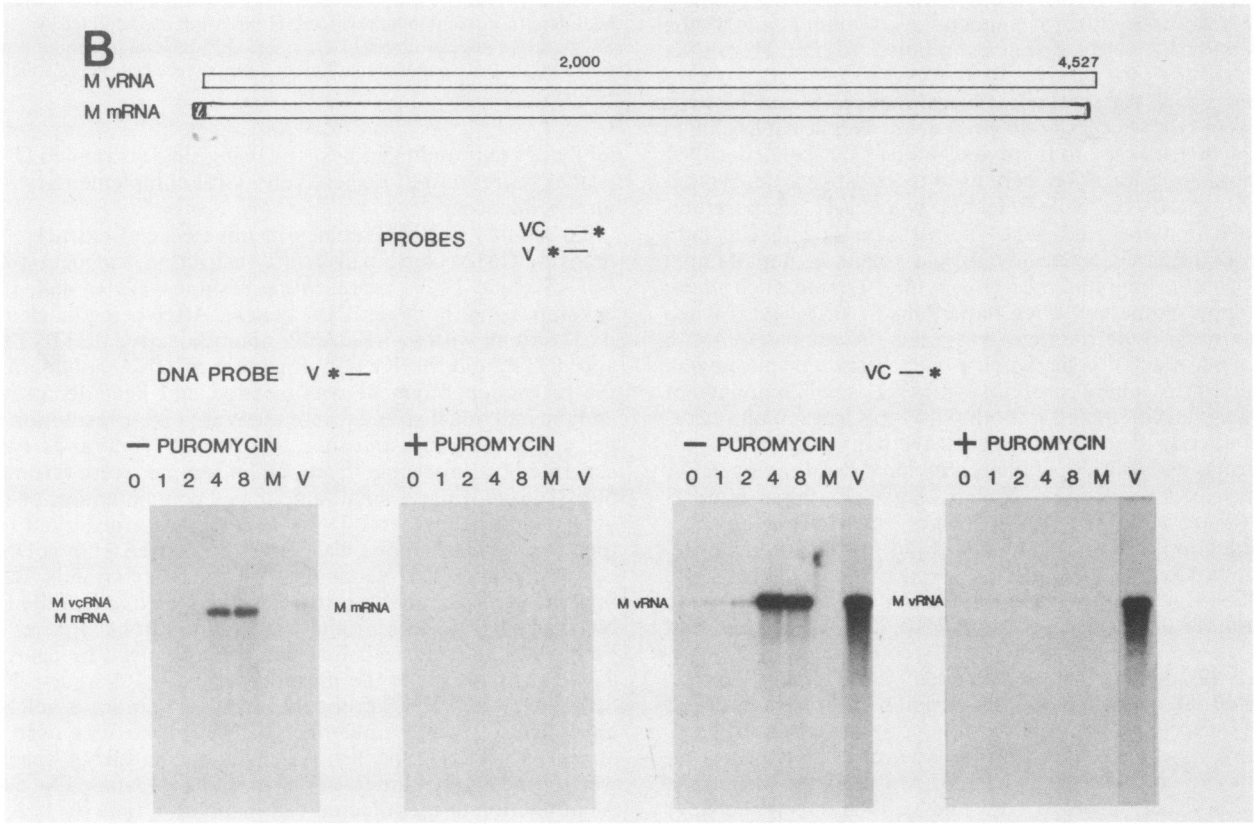
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were identified (Fig. 5A, bottom left panels). This was observed for extracts of infected cells incubated in the absence or, in smaller amounts, in the presence of puromycin (see above). In the absence of the protein synthesis inhibitor (but not when it was used), full-length S vcRNA was detected, in particular from 2 h postinfection. With the vcDNA probe, S vRNA was identified in extracts of infected cells incubated in the absence of puromycin but not in extracts incubated in the presence of the inhibitor. (The minor amounts of RNA that were observed to migrate ahead of the S vRNA were presumed to be S mRNA species that were detected owing to probe contamination). No vRNA was identified in the puromycin-treated cell extracts. Similar results were obtained for cells incubated in the presence or absence of 100 μg of cycloheximide per ml of culture fluid (data not shown).

To identify M RNA species, we recovered a 125-base-pair *Dde*I restriction fragment from SSH M clone 60-78 (16; i.e., residues 1820 to 1945, representing the middle of the coding region of the M gene). After being labeled at its 3′ termini with [α-^{32}P]dTTP, nonradioactive dCTP, dATP, and dGTP, plus the Klenow fragment of DNA polymerase, the restriction fragment was isolated and heat denatured, and the individual strands were recovered after resolution by gel electrophoresis. The bands were well separated (1 cm), and no evidence for cross contamination was obtained be rerunning samples. Each DNA preparation was used to identify cRNA species in the infected cell extracts (Fig. 5B). As expected, no vRNA species were found in mock-infected cell extracts (M lanes, Fig. 5B). In the extracts obtained from infected cells that were incubated in the absence of puromycin, RNA that migrated with the mobility corresponding to that of M vRNA was identified by both probes. For the vDNA probes, this presumably included both M mRNA species and similarly sized replicative intermediate M vcRNA species. For the vcDNA probe, the hybrids that were observed from 4 h postinfection presumably represented newly synthesized M vRNA species. In extracts obtained from cells incubated in the presence of puromycin, no viral-induced RNA species were detected (Fig. 5B). Similar results were obtained for extracts of infected cells incubated in the presence or absence of 100 μg of cycloheximide per ml of culture medium (data not shown).

DISCUSSION

Our results indicate that the M and S mRNA species of SSH bunyavirus are subgenomic in size and lack some 100 (S) or 60 (M) nucleotides at their 3′ termini by comparison with the sequences of the respective template vRNA species, although they possess 12 to 17 extra nucleotides at their 5′ termini. Thus, overall the M mRNA species is approximately 45 nucleotides shorter than the 4,527-nucleotide-long M vRNA species and presumptive replicative intermediate vcRNA species. The S mRNA species is some 85 nucleotides shorter than the S vRNA and presumptive replicative intermediate vcRNA species.

The site of transcription termination of the S mRNA species was located at two or three nucleotides beyond residue 886 of the vRNA. The sequence of the template RNA at this site is (from residue 880) UUGGGUGUUUUU (7). Thus, transcription appears to terminate just after a template GGGUG sequence and probably on a template oligonucleotide U tract. Direct evidence for the 3′ end sequence of the S mRNA species has not been obtained, so that whether there is a short 3′ homopolymeric sequence that may account for the reported binding of a proportion of the SSH S mRNA species to oligo(dT) cellulose (13) is not known. The principal S1 nuclease-protected species (B1, Fig. 2) identified in the analyses to determine the site of S mRNA transcription termination was shown by sequence analyses to be approximately 100 nucleotides shorter than the species corresponding to the complete vcRNA species (A1, Fig. 2). Two minor bands of S1 nuclease-protected product (A2 and B2, Fig. 2) were also identified but not analyzed owing to the small quantities of material recovered. They may represent hybrids that were partially degraded at their 5′ ends. For the M mRNA species, the transcription termination site was located some 60 nucleotides before the 5′ end of the template M vRNA sequence (i.e., in the region of template vRNA residue 4465). Because sequence analyses were not undertaken (owing to the low quantities of material recovered), the precise end of the M mRNA is not known. There is, however, no template UUGGGUGUUUUU sequence or similar sequence involving a U tract greater than two residues long or a U tract of two or more residues in this region of the template M vRNA sequence. There are in fact 10 U_5 tracts that occur in the M vRNA sequence (16). They are located between residues 806 and 3101 and include two that are U_6 sequences. Thus, a homopolymeric U_5 or U_6 tract does not appear to account for (at least) the M mRNA transcription termination. Also, there is no GGGUG sequence in the M vRNA species, let alone near the indicated transcription termination site (Fig. 6). However, there is considerable homology between the 3′ end sequences of the SSH S and M vcRNA species (Fig. 6). With a minimal number of gaps, some 55% of the nucleotides beyond the M gene product translation termination codon are similar in type and location to those of the S vcRNA species. It has been remarked previously (2) that, unlike their other noncoding or single gene product coding regions, the 3′-terminal 120 nucleotides of the S vcRNA of SSH and LAC viruses are highly conserved. This conserved sequence is much longer than either the 30-to-40 5′-conserved nucleo-

FIG. 5. Analyses of the time course of synthesis of SSH virus-induced S and M RNA species recovered from cells incubated in the presence or absence of puromycin. (A) A 243-base-pair *Hin*fI restriction fragment from SSH S clone 17 (7) was labeled at its 3′ termini and heat denatured, and the vDNA and vcDNA strands were recovered after gel electrophoresis (7; schematic). Some contamination of the vcDNA preparation with vDNA was observed when a sample was rerun, but not vice versa. The DNA preparations were used as probes in Northern hybridization analyses with RNA extracted from mock (M)- or virus-infected cells or purified virus (V) resolved by agarose gel electrophoresis in the presence of methyl mercury (3, 4). The infected-cell RNA samples came from cells infected with 50 PFU of SSH virus per cell incubated in the presence or absence of 100 μg of puromycin per ml of culture fluid (see the text) and harvested at 0, 1, 2, 4, and 8 h postinfection. The identities of the indicated S vcRNA, S mRNA, and S vRNA species were deduced from the specificities of the DNA probes and previous analyses which showed that S mRNA migrates ahead of S RNA (13, 17). (B) A 125-base-pair *Dde*I restriction fragment was obtained from SSH M clone 60-78 (16), end labeled at its 3′ termini, and strand separated, and the vDNA and vcDNA species were recovered by gel electrophoresis (7; schematic). Each was used in Northern hybridization analyses as described above. The identities of the RNA species were deduced from the specificities of the probes.

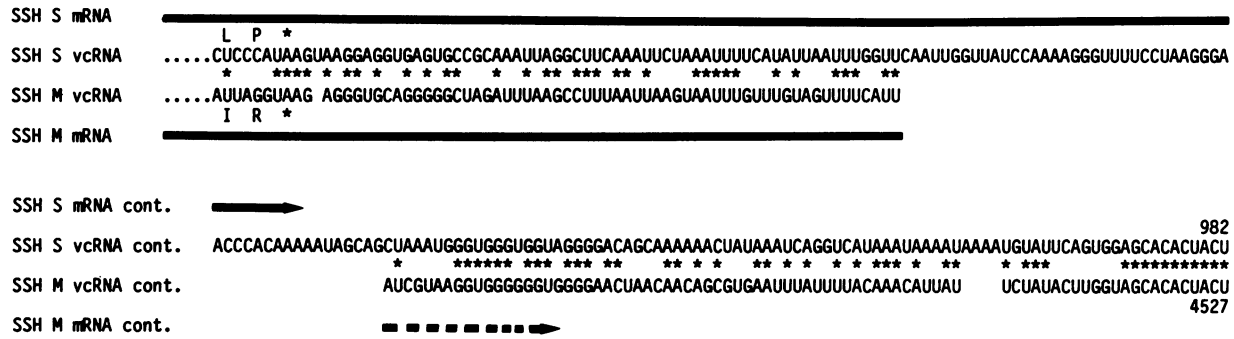

FIG. 6. Comparison of the end sequences of SSH M and S vcRNA species in relation to the indicated transcription termination sites. The M and S translation termination sites are shown (7, 16). By using a minimal number of gaps, the sequences of the two vcRNA species are aligned to present a best-fit arrangement. Homologous nucleotides are indicated by asterisks. The two terminal amino acids of the SSH S (N)- and M-coded gene products are indicated above their respective triplets. L, Leucine; P, proline; I, isoleucine; R, arginine.

tides of the vcRNA species of the two viruses or the lengths of the complementary ends of the S RNA species of either virus (2, 7), or that of SSH M RNA (16). The comparable conservation of sequences at the 3′ ends of the SSH and LAC S and SSH M vcRNA species is probably related to a functional requirement predictably involving transcription termination as well as end hydrogen bonding and recognition by a polymerase for vRNA replication and (in the 5′ vcRNA sequence) N protein for nucleocapsid formation. In conclusion, although there are homologous sequences between the SSH S and M vRNA species, the sites of transcription termination of their respective mRNA species do not appear to involve a common template sequence. Why, therefore, transcription is terminated at the indicated sites is not known. It is of interest to note, however, that S mRNA transcription terminates some 15 nucleotides before the beginning of a pyrimidine-rich sequence in the template RNA, a region that is conserved in the M vRNA sequence and that is apparently the site of M mRNA transcription termination.

Evidence has been obtained that indicates that the 5′ ends of both the S and M mRNA species have heterogeneous, 12- to 17-nucleotide-long terminal sequences with an indication of a preference for an A at the −1 (S and M mRNA) and a C at the −2 (M mRNA) position. The significance of the preference for AC in the −2 and −1 positions is not known; it may relate to sequences of preferred cellular RNA species that are scavenged for sequences to be used for bunyavirus mRNA synthesis. In this connection, it is of interest that not only does bunyavirus vcRNA synthesis proceed in the presence of actinomycin D and α-amanitin (33), but S mRNA synthesis can be detected in cells incubated in the presence of protein synthesis inhibitors. Presumably, therefore, unlike influenza virus, the bunyavirus mRNA transcription processes use preformed mRNA species that are already available in the cytoplasm of infected cells (because bunyaviruses have been reported to grow in enucleated cells; see reference 8). Direct evidence for this hypothesis has yet to be obtained.

The demonstration of S mRNA synthesis in virus-infected cells grown in the presence of inhibitors of protein synthesis confirmed our previous studies (33). Presumably, the inability to detect mRNA synthesis that was reported by other investigators (1, 29) is related to the sensitivities of the assays that were used and, possibly, lower multiplicities of infection. Our inability to detect M mRNA synthesis in puromycin- or cycloheximide-treated infected cells may also relate to the insensitivity of the assay we used, particularly if, as indicated in previous studies (33), there is significantly less M and L mRNA synthesis than S mRNA synthesis. If this is correct, the reason for the different regulation of the synthesis of the various bunyavirus mRNA species is not known.

LITERATURE CITED

1. **Abraham, G., and A. K. Pattnaik.** 1983. Early RNA synthesis in Bunyamwera virus-infected cells. J. Gen. Virol. **64**:1277–1290.
2. **Akashi, H., and D. H. L. Bishop.** 1983. Comparison of the sequences and coding of La Crosse and snowshoe hare bunyavirus S RNA species. J. Virol. **45**:1155–1158.
3. **Alwine, J. C., D. J. Kemp, and G. R. Starh.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. U.S.A. **74**:5350–5354.
4. **Bailey, J. M., and N. Davidson.** 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. **70**:75–85.
5. **Bishop, D. H. L., C. H. Calisher, J. Casals, M. T. Chumakov, S. Y. A. Gaidamovich, C. Hannoun, D. K. Lvov, I. D. Marshall, N. Oker-Blom, R. F. Pettersson, J. S. Porterfield, P. K. Russell, R. E. Shope, and E. G. Westaway.** 1980. Bunyaviridae. Intervirology **14**:125–143.
6. **Bishop, D. H. L., M. E. Gay, and Y. Matsuoko.** 1983. Nonviral heterogeneous sequences are present at the 5′ ends of one species of snowshoe hare bunyavirus S complementary RNA. Nucleic Acids Res. **11**:6409–6418.
7. **Bishop, D. H. L., K. G. Gould, H. Akashi, and C. M. Clerx-van Haaster.** 1982. The complete sequence and coding content of snowshoe hare bunyavirus small (S) viral RNA species. Nucleic Acids Res. **10**:3703–3713.
8. **Bishop, D. H. L., and R. E. Shope.** 1979. Bunyaviridae, p. 1–156. *In* H. Fraenkel-Conrat and R. Wagner (ed.), Comprehensive virology, vol. 14. Plenum Publishing Corp., New York.
9. **Bouloy, M., and C. Hannoun.** 1976. Studies on Lumbo virus replication. I. RNA-dependent RNA polymerase associated with virions. Virology **69**:258–264.
10. **Bouloy, M., and C. Hannoun.** 1976. Studies on Lumbo virus replication. II. Properties of viral ribonucleoproteins and characterization of messenger RNAs. Virology **71**:363–370.
11. **Bouloy, M., S. J. Plotch, and R. M. Krug.** 1978. Globin mRNAs are primers for the transcription of influenza viral RNA *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **75**:4888–4890.
12. **Bouloy, M., P. Vialat, M. Girard, and N. Pardigon.** 1984. A transcript from the S segment of the Germiston bunyavirus is uncapped and codes for the nucleoprotein and a nonstructural protein. J. Virol. **49**:717–723.
13. **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.
14. **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.
15. **Denhardt, D.** 1966. A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–652.
16. **Eshita, Y., and D. H. L. Bishop.** 1984. The complete sequence of the M RNA of snowshoe hare bunyavirus reveals the presence of internal hydrophobic domains in the viral glycoproteins. Virology **137**:227–240.
17. **Fuller, F., A. S. Bhown, and D. H. L. Bishop.** 1983. Bunyavirus nucleoprotein, N, and a non-structural protein, NS_S, are coded by overlapping reading frames in the S RNA. J. Gen. Virol. **64**:1705–1714.
18. **Fuller, F., and D. H. L. Bishop.** 1982. Identification of virus-coded nonstructural polypeptides in bunyavirus-infected cells. J. Virol. **41**:643–648.
19. **Gait, J. J., M. Singh, R. C. Sheppard, M. D. Edge, A. R. Greene, G. R. Heathcliffe, T. C. Atkinson, C. R. Newton, and A. F. Markham.** 1980. Rapid synthesis of oligodeoxyribonucleotides. IV. Improved solid phase synthesis of oligodeoxyribonucleotides through phosphotriester intermediates. Nucleic Acids Res. **8**:1081–1096.
20. **Gentsch, J. R., and D. H. L. Bishop.** 1978. Small viral RNA segment of bunyaviruses codes for final nucleocapsid protein. J. Virol. **28**:417–419.
21. **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.
22. **Hefti, E., and D. H. L. Bishop.** 1975. The 5′ nucleotide sequence of vesicular stomatitis viral RNA. J. Virol. **15**:90–96.
23. **Ihara, T., H. Akashi, and D. H. L. Bishop.** 1984. Noval coding strategy (ambisense genomic RNA) revealed by sequence analyses of Punta Toro phlebovirus S RNA. Virology **136**:293–306.
24. **Krug, R. M., B. B. Broni, and M. Bouloy.** 1979. Are the 5′ ends of influenza viral mRNAs synthesized *in vivo* donated by host mRNAs? Cell **18**:329–334.
25. **Maxam, A., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base specific chemical cleavages. Methods Enzymol. **65**:499–560.
26. **Obijeski, J. F., A. T. Marchenko, D. H. L. Bishop, B. W. Cann, and F. A. Murphy.** 1974. Comparative electrophoretic analysis of the virus proteins of four rhabdoviruses. J. Gen. Virol. **22**:21–33.
27. **Patterson, J. L., C. Cabradilla, B. P. Holloway, J. F. Obijeski, and D. Kolakofsky.** 1983. Multiple leader RNAs and messages are transcribed from the La Crosse virus small genome segment. Cell **33**:791–799.
28. **Patterson, J. L., B. Holloway, and D. Kolakofsky.** 1984. La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease. J. Virol. **52**:215–222.
29. **Patterson, J. L., and D. Kolakofsky.** 1984. Characterization of La Crosse virus small-genome transcripts. J. Virol. **49**:680–685.
30. **Pattnaik, A. K., and G. Abraham.** 1983. Identification of four complementary RNA species in Akabane virus-infected cells. J. Virol. **47**:452–462.
31. **Plotch, S. J., M. Bouloy, and R. M. Krug.** 1979. Transfer of 5′ terminal cap of globin mRNA to influenza viral complementary RNA during transcription *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **76**:1618–1622.
32. **Ranki, M., and R. F. Pettersson.** 1975. Uukuniemi virus contains an RNA polymerase. J. Virol. **16**:1420–1425.
33. **Vezza, A. C., P. M. Repik, P. Cash, and D. H. L. Bishop.** 1979. In vivo transcription and protein synthesis capabilities of bunyaviruses: wild-type snowshoe hare virus and its temperature-sensitive group I, group II, and group I/II mutants. J. Virol. **31**:426–436.
34. **Winter, G., S. Fields, M. J. Gait, and G. G. Brownlee.** 1981. The use of synthetic oligodeoxynucleotide primers in cloning and sequencing segment 8 of influenza virus (A/PR/8/34). Nucleic Acids Res. **9**:237–245.